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Patel, Kajal M.

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# **ROLE OF PEROXISOME PROLIFERATOR ACTIVATED-RECEPTOR $\gamma$ IN LUNG EPITHELIAL CELLS**

Submitted by

**Kajal M. Patel**

For the degree of Ph.D.  
of the University of Bath

2005

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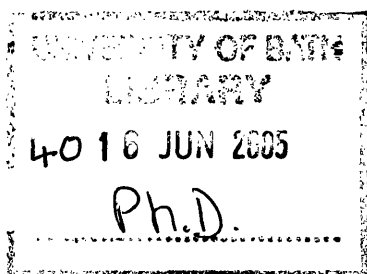
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## **Publications**

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## Abstract

The research presented within this thesis focuses on the role of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) in lung epithelial cells. PPARs are ligand activated transcription factors and have been implicated in diverse pathways and thus disease states. PPAR $\gamma$  ligands are reported to possess anti-inflammatory properties however there is much controversy over the mechanisms of action. The aim of this work was to clarify the role of PPAR $\gamma$  ligands in regulating inflammatory responses in A549 (human lung epithelial) cell line, and identify the signalling pathways and mechanism involved in doing this. Firstly this work characterises two phosphoinositide 3-kinase (PI3K) inhibitors and investigates the signalling pathways involved in the regulation of a pro-inflammatory enzyme, cyclooxygenase-2 (COX-2), by cytokines. Secondly this work investigates the effects of PPAR $\gamma$  ligands on the regulation of COX-2 in A549 cell line.

*In vitro* lipid kinase assays revealed that two PI3K inhibitors, LY294002 and wortmannin had differential selectivity to PI3K isoforms. The rank order of selectivity of LY294002 was p85-associated PI3K > PI3K-C2 $\alpha$  > PI3K-C2 $\beta$ , whereas the rank order of selectivity of wortmannin was p85-associated PI3K > PI3K-C2 $\beta$  and had no inhibitory effect on PI3K-C2 $\alpha$ . Due to the differential selectivity care must be taken when utilising these inhibitors to assess the role of PI3K pathway.

The pro-inflammatory cytokines TNF $\alpha$  and IL-1 $\beta$  activated the PI3K and mitogen-activated protein kinase pathway (MAPK) in a concentration and time-dependent manner. Both cytokines time-dependently induced the expression of COX-2 and microsomal prostaglandin E synthase (mPGES) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production. TNF $\alpha$ -induced COX-2 expression and PGE<sub>2</sub> was sensitive to the PI3K inhibitor LY294002, the MEK inhibitor PD98059 and the p38 inhibitor SB202190, but was insensitive to the PI3K inhibitor wortmannin and the PKC inhibitor Ro-32-0432. TNF $\alpha$  activated the NF $\kappa$ B pathway, as shown by I $\kappa$ B degradation, p65 phosphorylation and activation and NF $\kappa$ B gene reporter activity. These data suggest the role of PI3K, MAPK and NF $\kappa$ B in regulation of COX-2 expression by TNF $\alpha$ .

The synthetic PPAR $\gamma$  ligand troglitazone activated the PI3K and MAPK, whereas the endogenous PPAR $\gamma$  ligand, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), only activated the PI3K pathway. 15d-PGJ<sub>2</sub> had no detectable effects on COX-2, mPGES expression, or PGE<sub>2</sub> production. However, troglitazone induced time-dependent COX-2 expression, which was insensitive to PPAR $\gamma$  antagonists, but was abrogated by inhibitors of PI3K and the ERK MAP kinase pathway. Furthermore, troglitazone induced mPGES expression and PGE<sub>2</sub> production. Neither troglitazone nor 15d-PGJ<sub>2</sub> was able to convincingly activate NF $\kappa$ B in A549 cells. Further heterogeneity in the responses to troglitazone and 15d-PGJ<sub>2</sub> was observed in the regulation of gene expression as assessed by microarray analysis.

In summary, this study provides compelling evidence that troglitazone (like 15d-PGJ<sub>2</sub>) can exert functional effects independently of actions via PPAR $\gamma$ . Moreover, it identifies unique biochemical and functional actions of troglitazone that are not shared by 15d-PGJ<sub>2</sub>, which may influence the therapeutic potential of this compound in inflammatory settings.

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## Abbreviations

AA	Arachidonic Acid
Ab	Antibody
ADP	Adenosine Diphosphate
AF	Activation function
ANOVA	Analysis of Variance
AP	Activator Protein
APS	Ammonium Persulphate
ASM	Airway smooth muscle
ATF	Activating Transcription Factor
ATP	Adenosine Triphosphate
BADGE	Bisphenol A diglycidyl ether
BSA	Bovine Serum Albumin
Ca <sup>2+</sup>	Calcium <sup>2+</sup> ions
CBP	CREB binding protein
cPLA <sub>2</sub>	Cytosolic Phospholipase A <sub>2</sub>
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
cPGES	Cytosolic prostaglandin E synthase
CRE	cAMP Response Element
CREB	cAMP response element binding protein
DAG	Diacylglycerol
DBD	DNA binding domain
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DRIP	Vitamin D <sub>3</sub> receptor
ECL	Enhanced chemiluminescent lumigen
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular Signal Regulated Kinase

FBS	Foetal Bovine Serum
GSK	Glycogen synthase kinase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HETE	Hydroxyeicosatetraenoic Acid
HO-1	Hemeoxygenase-1
HPETE	Hydroperoxyeicosatetraenoic Acid
HRP	Horseradish peroxidase
hsp	Heat Shock Protein
IBD	Inflammatory Bowel Disease
ICAM	Intercellular Adhesion Molecule-1
ICE	IL-1 $\beta$ converting enzyme
IFN	Interferon
Ig	Immunoglobulin
IKK	Inhibitor of $\kappa$ B Kinase
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
I $\kappa$ B	Inhibitor of $\kappa$ B
IP-10	Interferon-Inducible Protein 10
IRAK	IL-1R1 associated kinase
JAK	Janus Kinase
JNK	<i>c-jun</i> N-terminal Kinase
kb	Kilobase
LBD	Ligand binding domain
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTB <sub>4</sub>	Leukotriene B <sub>4</sub>
LY294002	Specific PI3K inhibitor
mAb	Monoclonal Antibody
MAP Kinase	Mitogen Activated Protein Kinase
MAPKAPK	Mitogen Activated Protein Kinase Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase

MCP	Monocyte Chemotactic Protein
MEK	MAP Kinase ERK Kinase
MKK	Mitogen Activated Protein Kinase Kinase
mPGES	Microsomal prostaglandin E synthase
mRNA	Messenger Ribonucleic Acid
MSK	Mitogen and stress activated protein kinase
mTOR	Mammalian target of rapamycin
MWt	Molecular Weight
MyD88	Myeloid differentiation factor 88
NEMO	NF $\kappa$ B essential modulator
NFAT	Nuclear factor of activated T cells
NF-IL-6	Nuclear Factor Interleukin-6
NF $\kappa$ B	Nuclear Factor $\kappa$ B
NIK	Nuclear Factor $\kappa$ B Inducing Kinase
NO	Nitric Oxide
NSAID	Non-Steroidal Anti-Inflammatory Drug
OD	Optical Density
PBP	PPAR binding protein
PD98059	Selective MEK1/2 inhibitor
PDK	Phosphatidylinositol (3,4,5) Triphosphate Dependent Kinase
PG	Prostaglandin
PGDH	Prostaglandin dehydrogenase
15d-PGJ <sub>2</sub>	15-deoxy- $\Delta^{12,14}$ -prostaglandin J <sub>2</sub>
PH	Pleckstrin Homology
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-Kinase
PI3K-C2	Phosphatidylinositol 3-Kinase Class II
PKB	Protein Kinase B
PKC	Protein Kinase C
PLA <sub>2</sub>	Phospholipase A <sub>2</sub>
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate

PMSF	Phenylmethanesulphonyl Fluoride
PPAR	Peroxisome-Proliferator Activated Receptor
PPRE	Peroxisome Proliferator Response Element
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and Tensin Homologue Deleted from Chromosome 10
RA	Retinoic acid
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
RNA	Ribonucleic Acid
RXR	Retinoic Acid Receptor
SB202190	Specific p38 MAP Kinase inhibitor
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SH	Src Homology
SHIP	SH2 containing Inositol 5' polyphosphatase
sPLA <sub>2</sub>	Secretory Phospholipase A <sub>2</sub>
SRC	Steroid receptor coactivator
STAT	Signal Transducer and Activator of Transcription
TACE	TNF $\alpha$ converting enzyme
TATA	Adenine Thymine rich promoter sequence
TBS	Tris Buffered Saline
TEMED	N, N, N',N'-tetramethylethylene diamine
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TRAF	TNF-receptor associated factor
TRAP	Thyroid hormone receptor
TXA <sub>2</sub>	Thromboxane A <sub>2</sub>
TZD	Thiazolidinedione
VCAM	Vascular cell adhesion molecule



**One and three letter amino acid code**

<b>Amino acid</b>	<b>Three letter code</b>	<b>Single letter code</b>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

**1.**

# **INTRODUCTION**

## **1. INTRODUCTION**

### **1.1 History**

Since 1965, it has been known that a group of structurally diverse agents, collectively known as peroxisome proliferators, were able to promote an increase in the number of hepatic peroxisomes when administered to rodents (Hess et al., 1965). This phenomenon is associated with an increase in transcription of genes involved in fatty acid oxidation (de la Iglesia et al., 1996). The ability of peroxisome proliferators to activate gene transcription raised the possibility that they were acting through a receptor-mediated mechanism. This eventually led to the identification of peroxisome proliferator-activated receptor (PPAR) $\alpha$ , the first member of the PPAR subfamily to be identified (Issemann and Green, 1990). The group now also includes PPAR $\gamma$  (of which there are three isoforms  $\gamma$ 1,  $\gamma$ 2 and  $\gamma$ 3) (Fajas et al., 1998) and PPAR $\delta$ .

PPARs form an important subfamily of the nuclear hormone receptor superfamily and are ligand activated transcription factors. PPARs have been implicated in many diverse pathways including lipid and glucose homeostasis, control of cellular proliferation and differentiation, and thus play a role in many human diseases including diabetes, obesity, atherosclerosis, hypertension and cancer. Recently PPARs have also become an important focus in inflammatory diseases such as asthma (Benayoun et al., 2001) due to their anti-inflammatory effects in cells of the immune system (Jiang et al., 1998; Ricote et al., 1998a). However the mechanisms by which ligands of PPARs, and in particular PPAR $\gamma$ , induce anti-inflammatory

effects are not only poorly understood but also very controversial. This thesis focuses on the role of PPAR $\gamma$  in lung epithelial cells. Firstly I will set the scene by introducing the “inflammatory response” and the key players involved. Next I will describe some of the important signalling pathways that have been well recognised in mediating messages from receptors to inducing functional inflammatory responses. Finally I will describe PPARs, the signalling pathways and mechanisms they utilise and how they have been linked to inflammation.

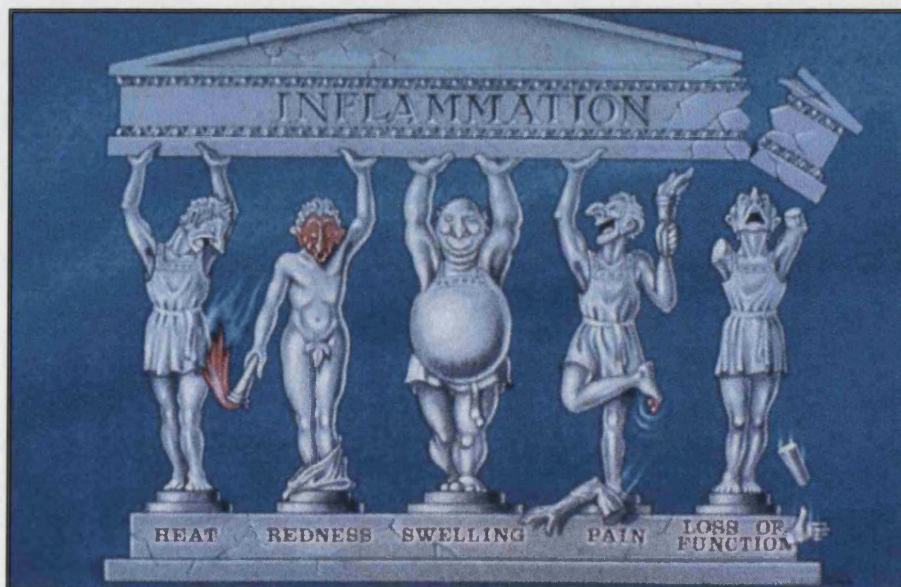
## **1.2 Inflammation**

### **1.2.1. Background**

Inflammation is a beneficial host response to foreign challenge or tissue injury that leads ultimately to the restoration of tissue structure and function. The inflammatory response requires innate immunity and, in some cases, an adaptive immune response, which are the two main integral components of the host's defence system. Innate immunity not only acts as the first line of defence against noxious material, but after recognition of an appropriate stimulus, it provides the necessary signals to instruct the adaptive immune system to mount a response. In turn, the adaptive response relies on the innate immune system to provide the necessary effectors, in the form of phagocytes and granulocytes, to deal with the initiating stimulus.

Inflammation is a reaction of the microcirculation that is characterized by the movement of serum proteins and leukocytes from the blood to the extravascular tissue. This movement is regulated by the sequential release of vasoactive and

chemotactic mediators, which contribute to the cardinal signs of inflammation-heat, redness, swelling, pain and loss of tissue function (Figure 1.1). Local vasodilation increases regional blood flow to the inflamed area and, together with an increase in microvascular permeability, results in the loss of fluid and plasma proteins into the tissues. Concomitantly, there is an upregulation of expression of adhesion molecules on endothelial cells and the release of chemotactic factors from the inflamed site, which facilitate the adherence of circulating cells to the vascular endothelium and their migration into the affected area. Phagocytic cells such as macrophages ingest foreign material and cell debris. They also release hydrolytic and proteolytic enzymes, and generate reactive oxygen species that eliminate and digest invading organisms. Finally, the injurious stimulus is cleared and normal tissue structure and function is restored. Thus the phases of inflammation have been loosely defined as acute, chronic and resolution.



**Figure 1.1 Cardinal signs of inflammation.** This cartoon depicts five Greeks representing the cardinal signs of inflammation-heat, redness, swelling, pain and loss of function, which was first described by Celsus more than 2000 years ago. Figure taken from (Lawrence et al., 2002).

There are many mediators that coordinate the initial events of acute inflammation (Table 1.1). Vasoactive amines, lipid-derived eicosanoids, cytokines and chemokines regulate vascular changes and inflammatory-cell recruitment (Larsen and Henson, 1983). Cell-adhesion molecules facilitate the movement of inflammatory cells from the peripheral circulation to the inflammatory site. Pro-inflammatory cytokines, such as tumour-necrosis factor (TNF) and interleukin-1 $\beta$  (IL-1 $\beta$ ), activate signalling pathways in endothelial cells that regulate the expression of these adhesion molecules to initiate the capture of circulating leukocytes (Shanley et al., 1995).

Mediator Class	Pro-inflammatory	Anti-inflammatory
Amines and peptides	Histamine, bradykinin	Adrenaline, noradrenaline
Lipid mediators	PGE <sub>2</sub> , PGI <sub>2</sub> , LTB <sub>4</sub> , LTC <sub>4</sub>	PGJ <sub>2</sub> , PGA <sub>1/2</sub> , lipoxins
Complement	C3a, C5a	C1q receptor
Cyclic nucleotides	cGMP	cAMP
Adhesion molecules	E-selectin, P-selectin, ICAM-1, VCAM-1	$\alpha_v\beta_3$ integrin, TSP receptor, PS receptor
Cytokines	TNF, IL-1 $\beta$ , IL-6	TGF- $\beta$ 1, IL-10
Chemokines	IL-8, GRO, MIP $\alpha$ , MCP1	-
Reactive oxygen species	O <sub>2</sub> <sup>-</sup> , OH $\cdot$ , H <sub>2</sub> O <sub>2</sub> , NO $\cdot$ , ONOO-	
Steroid hormones	-	Glucocorticoids

**Table 1.1. Mediators that regulate the acute inflammatory response** (Lawrence et al., 2002). Abbreviations: cAMP, cyclic adenosine 3,5 monophosphate; cGMP, cyclic 3,5 monophosphate; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LT, leukotriene; MCP1, monocyte chemotactic protein 1; MIP1 $\alpha$ , macrophage inflammatory protein 1 $\alpha$ ; NO $\cdot$ , nitric oxide; O<sub>2</sub><sup>-</sup>, superoxide anion radical; OH $\cdot$ , hydroxyl radical; ONOO-, peroxynitrite; PG, prostaglandin; PS, phosphatidylserine; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; TNF, tumour-necrosis factor; TSP, thrombospondin; VCAM-1, vascular cell adhesion molecule-1.

### 1.2.2. Key mediators of inflammation

#### *Reactive oxygen species*

Oxidant species are prevalent, at least transiently, in all active sites of metabolism, and are especially important in the pulmonary epithelium, where oxygen and oxidant exposure is continuous. A number of reactive species have been shown to affect cell function, including the oxygen species: superoxide anion radical ( $O_2^{\cdot-}$ ), hydroxyl radical ( $OH^{\cdot}$ ), hydrogen peroxide ( $H_2O_2$ ), and the nitrogen species: nitric oxide ( $NO^{\cdot}$ ), and peroxynitrite ( $ONOO^-$ ). Reactive oxygen and nitrogen species, known effectors of intracellular signalling in many cell types, are beginning to be recognized as intracellular signalling molecules in airway epithelium. Hydroxyl radical has also been implicated as an intracellular signalling molecule for intercellular adhesion molecule-1 (ICAM-1) expression in primary cultures of normal human bronchial epithelial cells and a human bronchial epithelial cell line (Krunkosky et al., 1996). Nitric oxide is produced by a small group of specific nitric oxide synthase (NOS) enzymes which includes the inducible form of NOS (iNOS). Nitric oxide (NO) appears to function in the intracellular regulation of secretion of mucus and ciliary motion in airway epithelial cells. NO can also be produced by the airway epithelium in response to primary inflammatory mediators, such as  $TNF\alpha$  and platelet activating factor (PAF), possibly serving to signal the upregulation of cellular processes that increase the inflammation observed in respiratory disease. Thus, nitric oxide may be considered a secondary mediator of inflammation produced by the effector tissue.



## Cytokines

Cytokines are extracellular signalling protein or peptides that act as a local mediator in cell-cell communication via activation of signalling pathways (which will be described in detail later) and thus play a pivotal role in the regulation of inflammation.  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$  can upregulate production of secondary mediators such as  $\text{IL-6}$ ,  $\text{IL-8}$ , granulocyte-macrophage colony-stimulating factor (GM-CSF), and insulin-like growth factor-1 (IGF-1).

## $\text{TNF}\alpha$

The tumour necrosis factor protein was identified in 1975 and named after its ability to kill tumour cells (Carswell et al., 1975). Subsequently, it was found to refer to two closely related cytokines,  $\text{TNF}\alpha$  and  $\text{TNF}\beta$  (or lymphotoxin- $\alpha$ ) which share receptors and play a part in a wide variety of human disease processes (Figure 1.2).

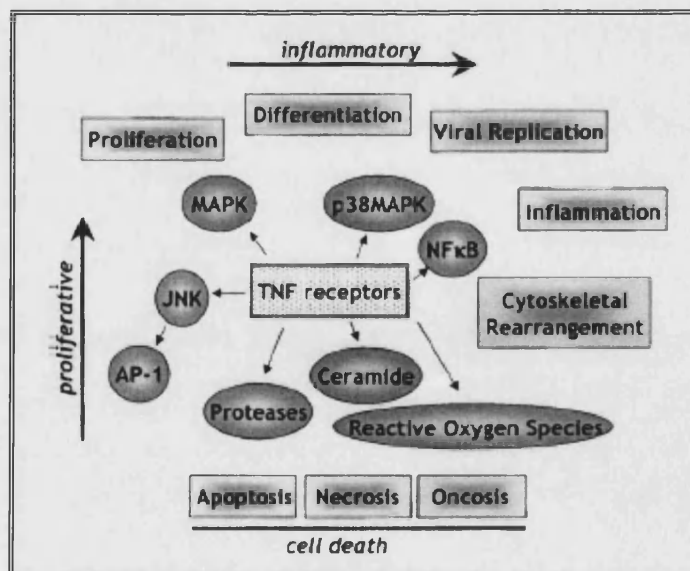


Figure 1.2. Major TNF mediated cellular responses (MacEwan, 2002)



TNF $\alpha$  is a member of a larger family of related ligands and is an immediate early gene. Its gene characteristics are outlined below:

- Multiple response elements in its promoter. These include binding sites for nuclear factor  $\kappa$ B (NF $\kappa$ B), activating protein (AP)-1, AP-2, specificity protein-1 (Sp1) and cAMP response element (CRE) and there is evidence of combinatorial activation as with COX-2.
- The presence of AREs in the 3'-UTR allowing post-transcriptional regulation via control of mRNA stability and translational rate.
- Expression in monocytes and macrophages, B and T lymphocytes and also lung epithelial cells
- Induced by a large number of diverse stimuli such as cytokines, growth factors, bacterial products and irradiation.

TNF $\alpha$  is first synthesised as a transmembrane (26kDa) precursor which can be biologically active in a juxtacrine manner. This precursor can then be cleaved by TNF $\alpha$  converting enzyme (TACE) into the 17kDa secreted form of TNF $\alpha$  which combine into homotrimers to bind to and activate its receptors.

#### *TNF $\alpha$ and airway diseases*

Asthma is perceived as a Th2 disease with a particular profile of cytokine release, which is thought to include IL-4 and IL-5. Increasing evidence indicates that other cytokines which are released are also associated with the inflammatory response that characterizes human asthma. One such mediator is TNF $\alpha$ , which has been implicated in asthmatic inflammation by a broad series of subcellular, *in vitro*, ex-vivo, *in vivo*

and genetic studies. TNF $\alpha$  is produced by many cells including macrophages, T-cells, mast cells and epithelial cells, but the principal source is the macrophage.

Both TNF $\alpha$  and IL-1 $\beta$  increase the expression of ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) on respiratory epithelial cells *in vitro*, and eosinophils show increased adherence to these cells after stimulation, although blocking experiments suggest that CD11/CD18 ( $\beta$ 2) integrins may play an important role in this adhesion (Moser et al., 1992; Godding et al., 1995). These effects are amplified in the presence of IL-5, perhaps via a CD18-dependent mechanism (Ebisawa et al., 1994). The TNF $\alpha$  induced increase in ICAM-1 also aids *in vitro* binding of activated T lymphocytes to airway smooth muscle cells, which is inhibited by cyclic AMP-dependent protein kinases (Ebisawa et al., 1994; Panettieri, Jr. et al., 1995). Thus, TNF $\alpha$  is associated with the up-regulation of adhesion molecules, and is able to facilitate inflammatory cell migration, particularly eosinophils in respiratory diseases (White et al., 1997). Furthermore, TNF $\alpha$  increases expression of the adhesion molecule, ICAM-1, levels of which are increased in the serum of chronic obstructive pulmonary disease (COPD) patients (Riise et al., 1994).

TNF $\alpha$  may activate macrophages to produce matrix metalloproteases (MMP)s (Mautino et al., 1997) and also stimulates bronchial epithelial cells to produce tenascin, an extracellular matrix glycoprotein (Harkonen et al., 1995). This data thus implicates TNF $\alpha$  in airway remodelling and fibrosis. In addition, TNF $\alpha$  appears to increase airway smooth muscle cell contractility (Thabut et al., 2002).

### *IL-1 $\beta$*

IL-1 is a highly inflammatory cytokine which can also upregulate host defences and act as an immunoadjuvant. It has two isoforms, IL-1 $\alpha$  and IL-1 $\beta$ , which belong to a larger, evolutionarily conserved, family which includes fibroblast growth factor (FGF) and IL-18. Both forms of IL-1 are synthesised as 31kDa precursor forms which require processing by specific proteases to produce a mature 17kDa secreted cytokine. Although the biological activities of IL-1 $\alpha$  and  $\beta$  are effectively indistinguishable, there are important differences between the two molecules.

ProIL-1 $\alpha$  is secreted by cells when they die and is then cleaved by extracellular proteases. It has been demonstrated that IL-1 $\alpha$  can be secreted in the absence of cell death *in vitro* (Watanabe and Kobayashi, 1994). However, the fact that IL-1 $\alpha$  is not commonly seen in the circulation or in body fluids, except during severe disease, means that release during cell death is likely to be the common mode of production (Watanabe and Kobayashi, 1994). In contrast IL-1 $\beta$  is released by living cells on stimulation and, like TNF $\alpha$  shares many features of an immediate early gene. The precursor form lacks biological activity and is converted to the mature form by the action of the cysteine protease (or caspase) IL-1 $\beta$  converting enzyme (ICE), although other inflammatory proteases may be able to catalyse the conversion in the absence of ICE (Fantuzzi et al., 1997).

IL-1 $\beta$  induces leukocytosis by the release of neutrophils from bone marrow, and also induces the release of many other cytokines such as IL-1, -2, -3, -4, -5, -6 and -8, regulated upon activation normal T-cell expressed and secreted (RANTES), GM-

CSF, interferon  $\gamma$  (IFN $\gamma$ ), TNF and platelet-derived growth factor (PDGF) from a variety of cells. It induces fibroblasts to proliferate, collagenase secretion, and increases synthesis of fibronectin and collagen.

IL-1 $\beta$  has been importantly implicated in driving the inflammatory response and resultant changes in airway smooth muscle (ASM) responsiveness in asthma. Furthermore, elevated levels of IL-1 $\beta$  have been detected in the bronchoalveolar lavage of patients with asthma (Broide et al., 1992; Sousa et al., 1996).

### ***Lipid mediators***

Lipid mediators, including prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids (HETEs) and PAF, are prevalent in inflammatory disease states. Both infiltrating inflammatory cells, and the epithelium produce these lipid mediators in response to a number of stimuli. In turn, these lipid products act both as primary and secondary mediators of inflammation. Of particular importance is the production of prostaglandins by the cyclooxygenase pathway, which is therefore discussed in greater detail in the following section.

## **1.3 Cyclooxygenase pathway**

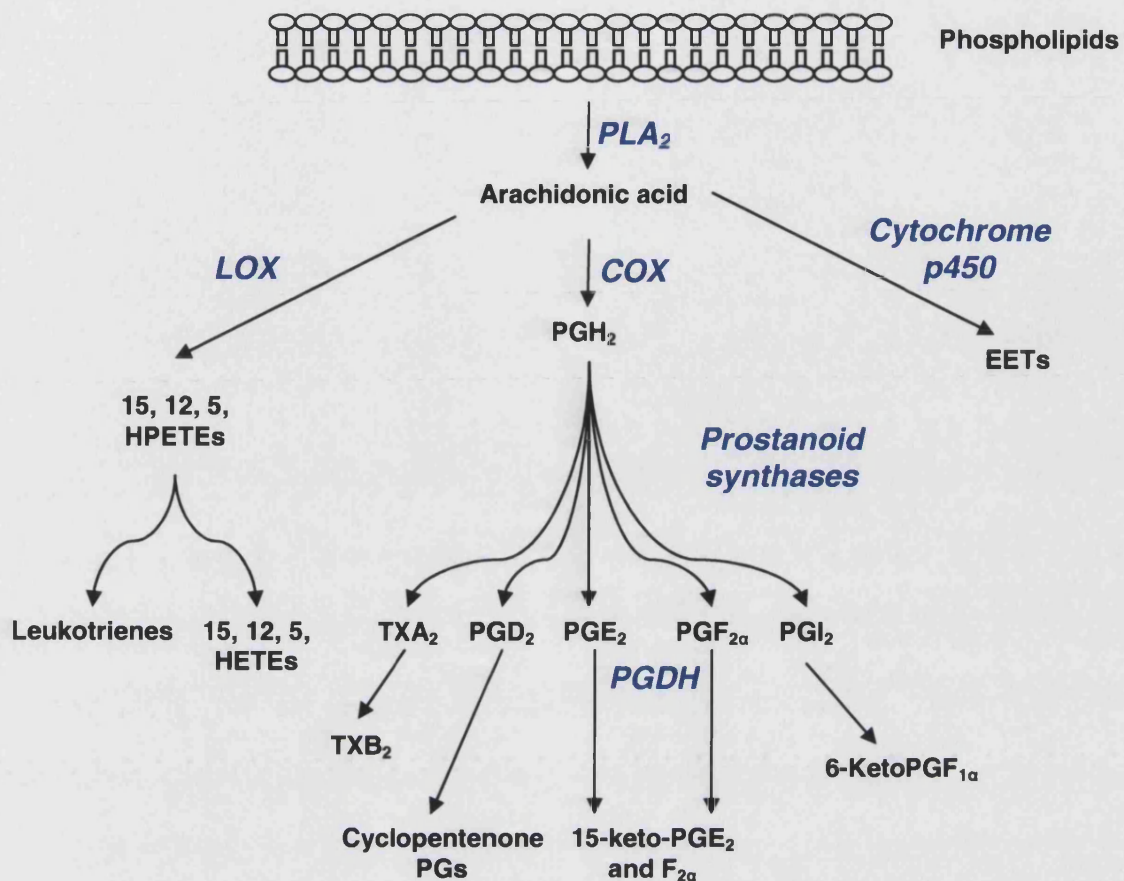
### **1.3.1. Background**

Prostanoids are mediators that have been implicated in all stages of inflammation. Indeed, the inhibition of prostanoid formation by non-steroidal anti-inflammatory drugs (NSAIDs) is the basis for their therapeutic as well as their side effects. The arachidonic acid pathway plays a vital role in production of lipid mediators, such as prostaglandins and leukotrienes, many of which are ligands of PPARs (described later).

Arachidonic acid liberation from phospholipids is a reaction catalysed by phospholipases A, C and D. The phospholipase A<sub>2</sub> (PLA<sub>2</sub>) family of enzymes are the most important with respect to prostaglandin production, and can be split into secretory or sPLA<sub>2</sub> (low MWt 14-18kDa), and cytoplasmic or cPLA<sub>2</sub> (high MWt 80-110 kDa). They represent a potential point of regulation of eicosanoid production and respond to agonist stimulation. There is evidence for some form of coupling between phospholipases and cyclooxygenases, although the mechanism for this has yet to be elucidated.

Free arachidonic acid can be metabolised by one of three pathways (these pathways are represented in Figure 1.3):

- Lipoxygenases
- Cytochrome p450
- Cyclooxygenases



**Figure 1.3. Pathways of Arachidonic Acid metabolism.** Schematic representation of the pathways involved in arachidonic acid metabolism and generation of eicosanoids. PLA<sub>2</sub> phospholipase A<sub>2</sub>; LOX lipoxygenase; COX cyclooxygenase; PG prostaglandin; HPETE hydroperoxyeicosatetraenoic acid; HETE hydroxyeicosatetraenoic acid; EET epoxyeicosatetraenoic acid; PGDH, 15-hydroxy-PG dehydrogenase

### 1.3.2. Lipoxygenases

Lipoxygenases insert an oxygen molecule into the twenty carbon ring of arachidonic acid and are numbered after the target carbon atom with 5-, 12-, and 15-lipoxygenase being the major enzymes relevant to gastrointestinal function. The ultimate products of lipoxygenase (LOX) activity are the leukotrienes, lipoxins and hydroxy fatty acids such as the hydroperoxyeicosatetraenoic (HPETEs) and hydroxyeicosatetraenoic (HETEs) acids.

### **1.3.3. Cytochrome p450**

The cytochrome p450 pathway of arachidonic acid metabolism represents a reaction where a single oxygen atom is inserted resulting in epoxy and ( $\omega/\omega^{-1}$ ) derivatives (Capdevila et al., 1981). Less is known about the functional importance of this pathway.

### **1.3.4. Cyclooxygenase-2 (COX-2)**

Arachidonic acid can be metabolised by the rate limiting enzyme, COX to prostaglandin  $H_2$  (PGH<sub>2</sub>). This is further metabolised by other enzymes to PGs, prostacyclin and thromboxane A<sub>2</sub> (Vane et al., 1994). COX enzymes catalyse the conversion of arachidonic acid to cyclic endoperoxides which serve as intermediates in the generation of prostaglandins – firstly by inserting two oxygen molecules to yield PGG<sub>2</sub> (cyclooxygenase activity) and then reducing this intermediate to give PGH<sub>2</sub> (peroxidase activity). PGH<sub>2</sub> is subsequently converted to a variety of eicosanoids, arachidonate derivatives based around a 20 carbon structure (eicosa is Greek for twenty), that include the prostaglandins and thromboxane A<sub>2</sub>. Which eicosanoids are produced is dependent on the environment, as well as the enzymatic machinery, of particular cells.

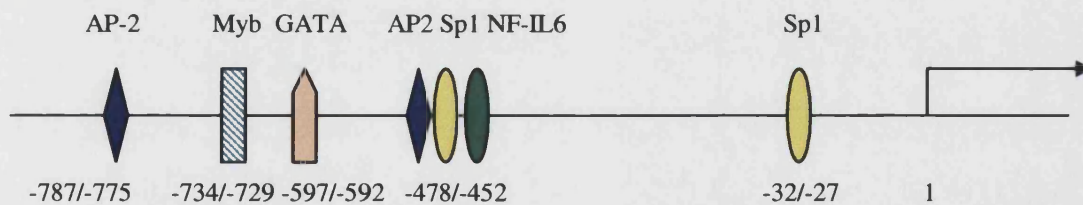
There are two well characterised isoforms of COX, COX-1 and COX-2. COX-1 which is the constitutive “housekeeping” isoform, COX-2 is an inducible isoform that can be upregulated by growth factors, cytokines and lipopolysaccharide in a wide variety of cells (Mitchell et al., 1994). Upregulation of COX-2 is a hallmark feature of many inflammatory diseases and thus understanding the mechanisms

involved in regulating this enzyme may prove important in identifying potential therapeutic targets for inflammatory diseases such as asthma, rheumatoid arthritis and inflammatory bowel disease. Recently, a third COX isoform has also been described (Botting, 2000). This enzyme is thought to be a variant of COX-2 induced by high concentrations of NSAID and was shown to be highly sensitive to inhibition by paracetamol. Thus it is thought that COX-3 may be a product of the same gene that encodes COX-2, but has different molecular characteristics (Botting, 2000).

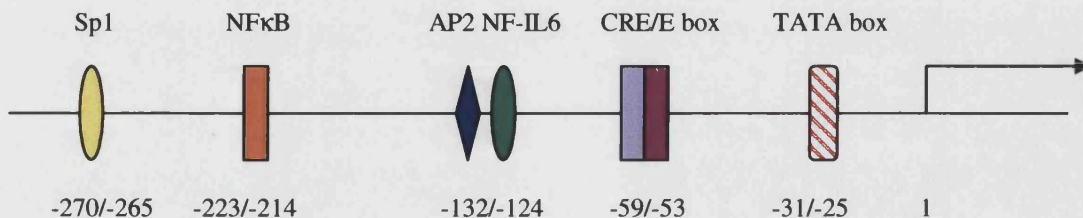
### *COX gene expression*

COX-1 is constitutively expressed in many tissues and cells in the animal species. The 5'-flanking region of the human COX-1 gene has multiple transcription start sites, does not possess a canonical TATA or CAAT box, and is GC-rich (Figure 1.4).

#### **Human COX-1 promoter**



#### **Human COX-2 promoter**



**Figure 1.4. Regulatory elements in the human COX-1 and COX-2 promoter.**



These features are consistent with those of a housekeeping gene. The 5'-flanking region of the COX-1 gene possesses several putative binding sites for transcriptional activators. Sp1 has been shown to contribute to constitutive expression of COX-1 in human umbilical vein endothelial cells by electrophoretic mobility shift assays (EMSA) (Xu et al., 2000). The regulation of COX-2 gene expression can be attributed to many regulatory elements, such as the nuclear factor  $\kappa$ B (NF $\kappa$ B) site, NF-IL6 motif, CRE and E-box (Figure 1.4).

The ATF/CRE has been identified as one of the most essential regulatory elements in the COX-2 promoter region. It has been shown that homo- or heterodimers of c-fos, c-jun, ATF families of bZIP proteins and the cAMP response element binding protein (CREB) can bind to this element and activate the expression of COX-2 (Xie and Herschman, 1996). Activation at the NF-IL6 element is most often associated with C/EBP transcription factors. There are several members of the C/EBP transcription factor family including C/EBP $\alpha$ , C/EBP $\beta$  and C/EBP $\delta$ . Many studies on the expression of COX-2 have implicated the transcription factor NF $\kappa$ B in signalling (Ghosh et al., 1998).

### **1.3.5. Prostaglandin E synthases**

Specific downstream prostanoid synthases dictate which individual PGs will be synthesised following generation of endoperoxides by COX. There is also evidence that these prostanoid synthases can couple with either COX-1 or COX-2. One of the best known and most well studied prostaglandins is PGE<sub>2</sub> which requires the enzyme prostaglandin E synthase (PGES) for its synthesis from COX-generated cyclic

endoperoxides. An inducible microsomal PGES (m-PGES) couples predominantly with COX-2 (Jakobsson et al., 1999; Murakami et al., 2000), whereas a constitutively and widely expressed cytosolic PGES (c-PGES) is thought to be coupled to COX-1 (Tanioka et al., 2000). Both these enzyme activities are dependent on reduced glutathione (GSH). After treatment with IL-1 $\beta$ , mPGES-1 protein expression has been shown to be induced in A549 cell line (Jakobsson et al., 1999). The induction of mPGES-1 expression and PGE<sub>2</sub> production stimulated by IL-1 $\beta$  in A549 cells has been demonstrated (Thoren and Jakobsson, 2000) to be in concert with the induction of COX-2 expression. The large amounts of PGE<sub>2</sub> produced at the inflammation site by the coupling of COX-2 and mPGES synthase may be involved in the progression of inflammation.

### **1.3.6. Prostaglandins**

Prostaglandins are small lipid molecules that regulate numerous processes in the body, including kidney function, platelet aggregation, neurotransmitter release and modulation of immune function. Once synthesised, prostaglandins cannot be stored and act as local paracrine, autocrine or intracrine mediators. They are involved in a multiplicity of physiological responses and mediate the effects of COX expression.

As previously mentioned PGE<sub>2</sub> is an important mediator of inflammation and is the most well studied PGs. PGE<sub>2</sub> is produced by many cells and exerts its effects by binding to one (or a combination) of its four subtypes of receptors (EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub>). The receptors are rhodopsin-type receptors containing seven hydrophobic

transmembrane domains, coupled through their intracellular sequences to specific G proteins with different second messenger signalling pathways. The regulation of expression of the various subtypes of EP receptors on cells by inflammatory agents, or even PGE<sub>2</sub> itself, enables PGE<sub>2</sub> to affect tissues in a very specific manner (Harris et al., 2002a). Recently, several reports have documented the expression of functional EP receptors (EP<sub>1</sub>, EP<sub>3</sub> and EP<sub>4</sub>) on the nuclear membranes of cells (Guan et al., 1998; Bhattacharya et al., 1999). For example, EP<sub>3</sub> and EP<sub>4</sub> have been localized to the nuclear envelope of endothelial cells. These receptors are functional, because they can modulate transcription, including that of the gene encoding iNOS (Bhattacharya et al., 1999). The discovery of nuclear EP receptors provides an additional level of complexity to the PGE<sub>2</sub> mediated regulation of cells, and further study is necessary to determine the processes that govern the expression and function of the nuclear-localized receptors.

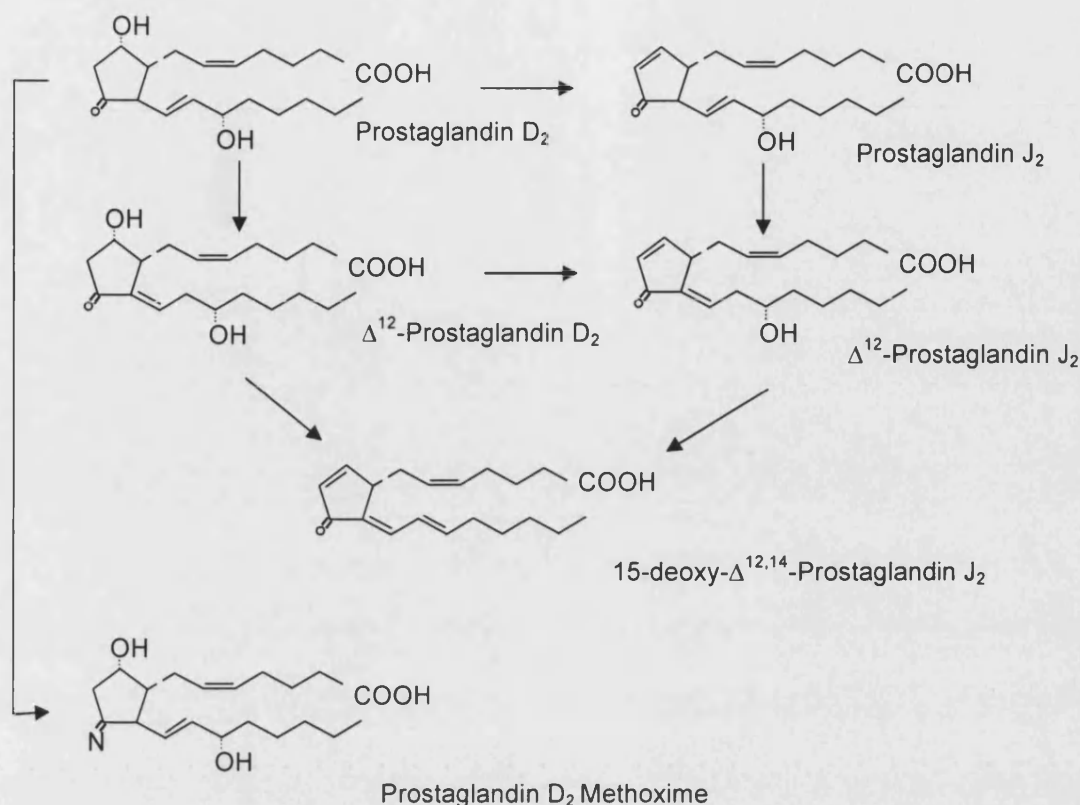
Other PG membrane receptors include DP, FP, IP and TP, based on their sensitivity to the primary other prostanoids PGD<sub>2</sub>, PGF<sub>2α</sub>, PGI<sub>2</sub> and TXA<sub>2</sub> respectively. The molecular cloning of the eight subtypes of prostanoid receptors has enabled the homogenous expression of each type of receptor in cultured cells and thus, an analysis of their ligand bindings and signal transducing properties. Table 1.2 summarizes the characteristics of the prostanoid receptors.

Eicosanoid	Receptor	Effect
PGE <sub>2</sub>	EP1	↑IP3/DAG
	EP2	↑cAMP
	EP3	↓Gi/cAMP
	EP4	↑cAMP
PGF <sub>2α</sub>	FP	↑IP3/DAG
TXA <sub>2</sub>	TP	↑IP3/DAG
PGI <sub>2</sub>	IP	↑cAMP
	PPAR $\delta$	
PGD <sub>2</sub>	DP	↑cAMP
PGJ <sub>2</sub>	PPAR $\gamma$	Transcriptional
15dPGJ <sub>2</sub>	PPAR $\gamma$	Transcriptional

Table 1.2. Summarises the receptors and some biochemical effects of various eicosanoids.

### *Regulation of inflammation by cyclopentenones*

In contrast to conventional PGs that act on G protein-coupled cell surface receptors, cyclopentenone PGs, such as  $\Delta^{12}$ -PGJ<sub>2</sub> and 15d-PGJ<sub>2</sub>, have no cell surface receptor. The J series of cyclopentenone PGs, once thought to comprise of inactive degradation products of PGD<sub>2</sub>, is now well established as regulating diverse processes, such as adipogenesis, inflammation and tumorigenesis. 15d-PGJ<sub>2</sub> is the end-product metabolite of PGD<sub>2</sub> (Figure 1.5) and is produced by a variety of cells, including mast cells, T cells, platelets and alveolar macrophages.



**Figure 1.5. Schematic representation of PGD<sub>2</sub> derivatives.** This demonstrates the metabolism of PGD<sub>2</sub> to both the J series of cyclopentenone prostaglandins, as well as the conversion to the stable methoximated derivative

The exact mechanism of entry of 15d-PGJ<sub>2</sub> into cells is unknown, but it is possible that 15d-PGJ<sub>2</sub> enters by an active transport system similar to those described by Narumiya et al. (1986) group for other cyclopentenone prostaglandins (e.g.  $\Delta^{12}$ -PGJ<sub>2</sub>). Alternatively, once synthesised 15d-PGJ<sub>2</sub> may act within the same cell. An additional cryptic mechanism allows transport of the cyclopentenone prostaglandins into the nucleus, where they affect gene transcription and thus exert a variety of biological actions including induction of stress response, inhibition of cell-cycle progression, suppression of viral replication, cell differentiation and development. Although it has never been shown directly that 15d-PGJ<sub>2</sub> uses these transporters, its similar structure to the other PGs makes this mechanism feasible. It is possible that

locally produced PGD<sub>2</sub> could enter a cell by an anionic transporter molecule and then be degraded to 15d-PGJ<sub>2</sub> once inside the cytoplasm.

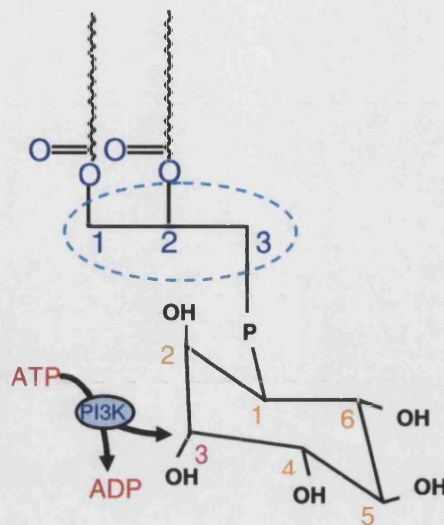
Gilroy et al. (1999), presented evidence that suggested that COX-2 may have anti-inflammatory properties. This study demonstrated that COX-2 may be pro-inflammatory during the early phase of a carrageenin-induced pleurisy, dominated by PMN cell-dominated phase, but may aid resolution by generating an alternative set of anti-inflammatory PGs (Gilroy et al., 1999).

#### **1.4. Signalling pathways**

Signalling pathways are vital components in inducing functional effects downstream of receptor binding. This next section will introduce the main signalling pathways which have been investigated in this thesis and that have been known to be activated downstream of cytokine receptors and have been shown to be important in the induction of COX-2. These include the phosphoinositide 3-kinase (PI3K), the mitogen-activated protein kinase (MAPK) and transcriptional pathways (NFκB and AP-1).

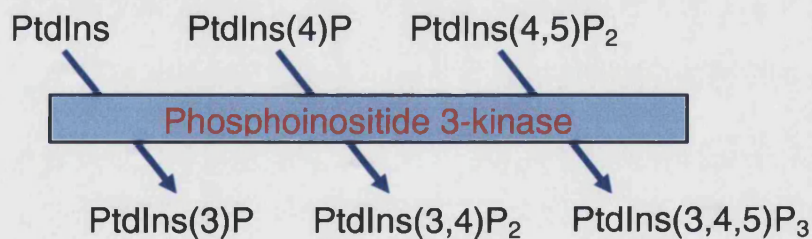
##### **1.4.1. Phosphoinositide 3-kinase (PI3K)**

PI3K mediates the formation of D-3 phosphoinositide lipids by transferring the terminal phosphate of ATP to the D-3 position of the inositol head group of phosphoinositide lipids (Figure 1.6).



**Figure 1.6.** Transfer of terminal phosphate of ATP to D-3 position of the inositol head group of phosphoinositide lipids.

PI3K can thus potentially produce three products: phosphatidylinositol (3)-phosphate [PtdIns(3)P], phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P<sub>2</sub>] and phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P<sub>3</sub>] (Figure 1.7) (DuBois et al., 1998; Vanhaesebroeck and Waterfield, 1999).

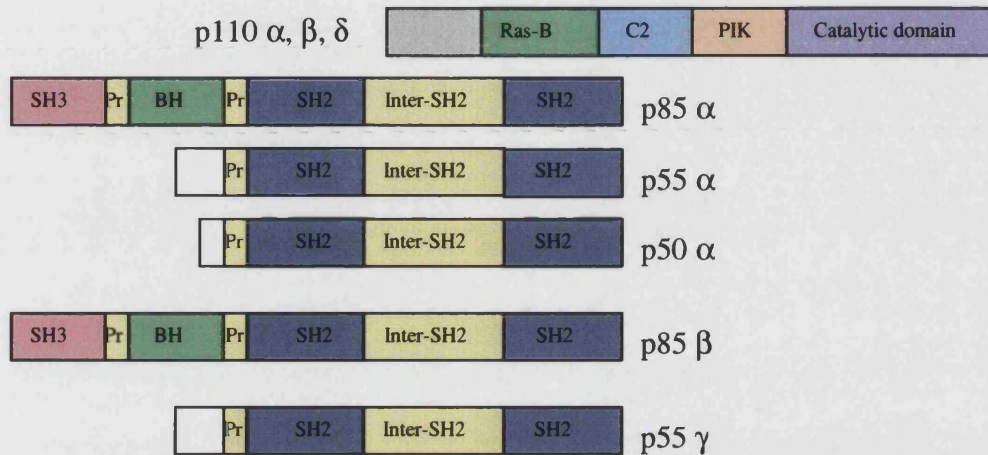


**Figure 1.7.** Formation of phosphoinositide lipids by PI3K.

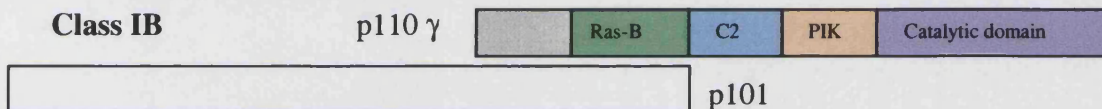
PI(3)P is constitutively present in eukaryotic cells and its levels are largely unaltered upon cellular stimulation. In contrast, PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> are generally absent from resting cells, but their intracellular concentration rises markedly upon stimulation via a variety of receptors suggesting a second messenger

function. There are multiple isoforms of PI3K in mammalian cells and these are subdivided into three classes on the basis of the *in vitro* lipid substrate specificity, structure (Figure 1.8) and likely mode of regulation (Koyasu, 2003).

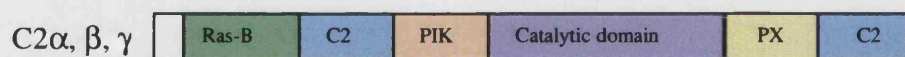
### Class IA



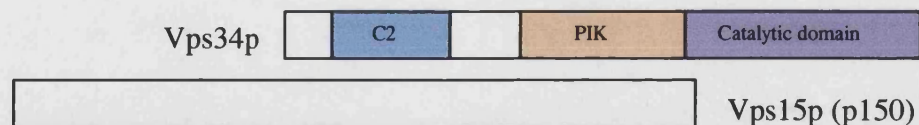
### Class IB



### Class II



### Class III



**Figure 1.8. Structural characteristics of the PI3K family.** The PIK is a helical domain that is found in lipid kinases but not protein kinases. C2 domains bind phospholipids in a calcium-dependent and – independent manner; those of PI3Ks do so in a calcium-independent fashion and recruit PI3Ks to the membrane. The SH2 domain of p85 $\alpha$ , p55 $\alpha$ , p50 $\alpha$ , p85 $\beta$  and p55 $\gamma$  constitutively interacts with the N-terminal domain of p110 $\alpha, \beta, \delta$ . Dual SH2 domains bind to tyrosine-phosphorylated adaptor proteins, leading to activation of the kinase activity of the p110 subunits. p101 specifically interacts with the N-terminal domain of p110 $\gamma$ . PX domains are known to bind PI(3)P and PI(3,4)P<sub>2</sub> and may recruit the class II PI3K to the membrane. Functions of the Ras-binding domain (Ras-B), SH3 and Bcr domains and the praline-rich regions (Pr) are not fully understood. Vps34p, the class III PI3K, forms a complex with a serine/threonine kinase Vps15p.



### ***Class I***

The Class I PI3K phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P<sub>2</sub>, resulting in the formation of PtdIns(3)P, PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> *in vitro*, whereas PtdIns(4,5)P<sub>2</sub> is the main substrate *in vivo*. They also interact with Ras and form heterodimeric complexes with adaptor proteins that link them to different upstream signalling events. The prototypical class IA PI3K is a heterodimer consisting of the 85kDa regulatory subunit (responsible for protein-protein interactions either via protein tyrosine phosphate-binding SH2 domains or SH3 domains and/or proline-rich regions) and a catalytic 110kDa subunit. Mammals have three genes for the p110 subunits, which encode p110 $\alpha$ , p110 $\beta$  and p110 $\delta$ . The existence of multiple isoforms of both components, which do not appear to preferentially associate, means that there is considerable scope for specific variation between tissues and with association to different receptor tyrosine kinases. The class IB PI3K are stimulated by G protein  $\beta\gamma$  subunits and do not interact with the SH2-containing adaptors that bind class IA PI3K. Instead, the first identified member of this family, p110 $\gamma$ , associates with a unique p101 adaptor molecule.

### ***Class II and Class III***

Class II PI3Ks are large proteins that have a catalytic domain that is 45-50% homologous to Class I PI3K. Class II PI3K also have a C-terminal region that has homology to the C2 domains that mediate calcium/lipid binding in classical protein kinase C isoforms (Cantrell, 2001). Class II PI3K preferentially phosphorylate PtdIns and PtdIns(4)P *in vitro*, whereas the Class III PI3K utilise only PI as a

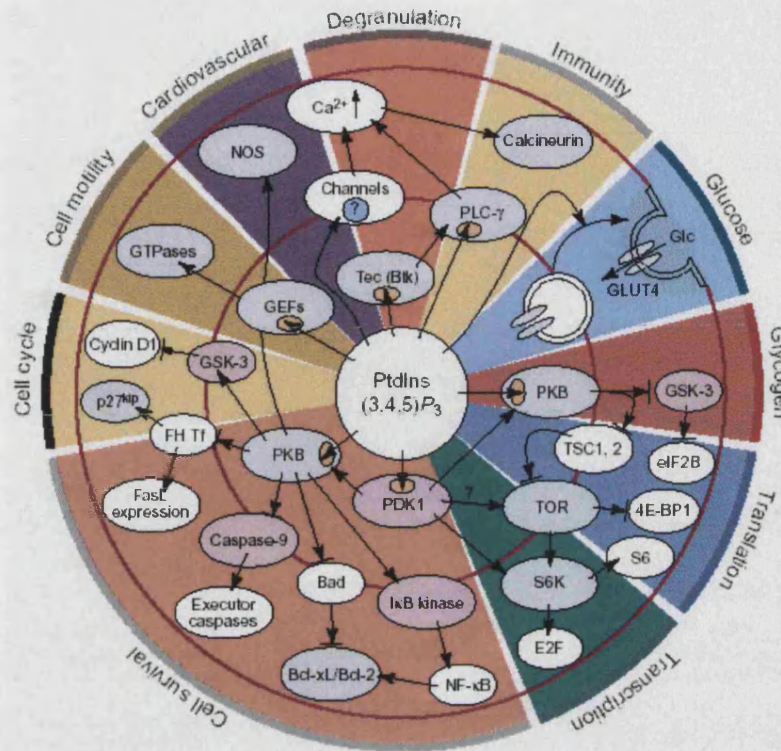
substrate. The mammalian homologue of Class III PI3K is likely to be the main source of PtdIns(3)P.

### ***Phosphatases***

The PI3K family mediate various biological effects through the action of the D-3 phosphorylated phosphoinositides second messengers. The levels of the second messenger are regulated by PTEN (phosphatase and tensin homolog) and SHIP (Src-homology-2 containing inositol 5'-phosphatase) that can specifically hydrolyze the D-3 and D-5 phosphates respectively from the lipid products and thus control the level of PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>.

### ***Downstream targets and functions***

A number of proteins have been identified that directly bind PtdIns(3,4,5)P<sub>3</sub> and/or PtdIns(3,4)P<sub>2</sub> via pleckstrin homology (PH) domains, which are lipid binding domains, including protein kinase B (PKB/Akt), PtdIns(3,4,5)P<sub>3</sub>-dependent protein kinase-1 (PDK-1), Bruton's tyrosine kinase, various PLC isoforms and exchange factors for the ADP-ribosylation factor family of GTP-binding proteins (Figure 1.15). Moreover, the D-3 phosphoinositide lipids have been linked to the triggering of a diverse array of cellular responses including cell survival, mitogenesis, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling, superoxide production as well as actin polymerisation and chemotaxis (Figure 1.9).



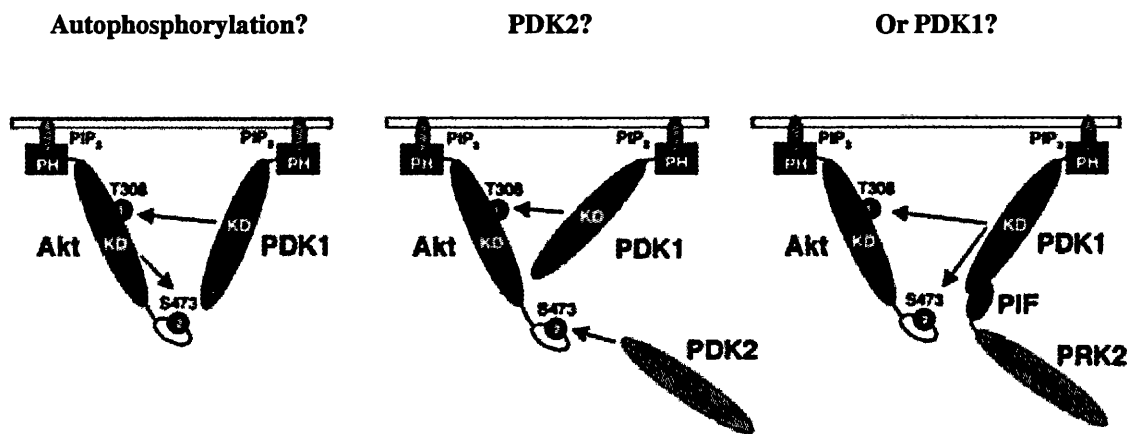
**Figure 1.9 Downstream targets and functions of PI3K.** Phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P<sub>3</sub>] produced by class I PI3Ks recruits proteins that contain a pleckstrin homology (PH) domain, including protein kinase B (PKB), phosphoinositide-dependent kinase 1 (PDK1), guanine nucleotide exchange factors (GEFs), protein tyrosine kinases of the Tec family [e.g. Bruton's tyrosine kinase (Btk)], phospholipase Cγ (PLC-γ) and many more. PI3K exerts control on most cellular processes and although this control is central, the signalling pathways depicted intersect with parallel, non-PI3K-dependent mechanisms (not shown) (Wymann et al., 2003). Abbreviations: 4E-BP1, eukaryotic initiation factor 4E binding protein 1; Glc, glucose; GLUT4, glucose transporter 4; IκB, inhibitor of NFκB; NFκB, nuclear factor κB; TSC1, hamartin; TSC2, tuberlin.

### PKB

Of these downstream effectors of PI3K, PKB is of particular interest. PKB is also known as Akt and is the prototype of a family of kinases that includes three known members, PKBα, PKBβ and PKBγ. It is a serine/threonine kinase that is itself activated by two distinct phosphorylation events, and which probably both involve the upstream kinase PH domain-containing kinase PDK-1. The binding of PtdIns(3,4,5)P<sub>3</sub> to PKBα PH domain brings this kinase to the plasma membrane, where it is phosphorylated on Thr308 in the kinase activation loop and on Ser473 in

a hydrophobic region of the C-terminus. The upstream kinase that targets Thr308 is a ubiquitously expressed kinase, PDK1 (Alessi et al., 1997a; Alessi et al., 1997b; Stephens et al., 1998). Three models have been proposed for the phosphorylation of the hydrophobic motif in PKB (Figure 1.10). Phosphorylation of Thr308 and Ser473 residues on PKB leads to its activation.

PKB has now been demonstrated to phosphorylate a number of proteins, particularly those involved in regulating glucose metabolism and cell survival, such as GSK-3. In particular, PDK-1 and PKB as well as the PI3K homologue, mammalian target of rapamycin (mTOR), contribute to the activation of p70<sup>S6</sup> kinase, an enzyme thought to initiate protein synthesis.



**Figure 1.10. Three models for the phosphorylation of the hydrophobic motif in Akt/PKB.** Left panel, Ser473 in the hydrophobic motif of Akt/PKB is regulated by autophosphorylation as a consequence of the PDK-1 step (i.e. Thr308 phosphorylation). Middle panel, a putative PDK2 functions as a heterologous kinase for Ser473 phosphorylation. Right panel, interaction of proteins (e.g., PRK2) with PIF-containing sequences with the PIF-binding pocket in PDK-1, converts PDK-1 into a Ser473 kinase (Toker and Newton, 2000). KD, kinase domain; PIF, PDK1 interacting factor; PRK2, protein kinase C-related kinase 2.

***Approaches utilised to dissect PI3K function***

There are three approaches by which the role of PI3K in functional responses can be defined-

1. *Pharmacological*- Wortmannin and the structurally unrelated inhibitor LY294002 have high selectivity for PI3Ks (Arcaro and Wymann, 1993; Vlahos et al., 1994) and have facilitated experiments on the role of PI3K in various cellular responses *in vitro* (Walker et al., 2000), but it has been difficult to study the function of this enzyme family *in vivo*. The first *in vivo* evidence for the role of PI3Ks in the immune system came from studies in which rats were exposed to wortmannin. These studies indicated that wortmannin is a potent immune suppressor, but that it is also highly toxic (Gunther et al., 1989a; 1989b). Since wortmannin and LY294002 cannot discriminate between different isoforms of PI3K, and as PI3Ks are crucial for all organ systems, genetic approaches based on gene-manipulated mouse systems has provided a breakthrough in elucidating the *in vivo* role of the individual PI3K enzymes.
2. *Molecular approaches*- There are other methods of inhibiting PI3K such as molecular methods like micro-injection of antibodies or transferring dominant negative forms of PI3K into cells (Benistant et al., 2000; Sasaki et al., 2000; Stein and Waterfield, 2000; Weaver and Ward, 2001). Similar transfer methods can be used to manipulate PI3K by causing its over-expression, and these may be placed under the control of an environmental agent such as an antibiotic in the culture media (Craddock et al., 1999).
3. *Genetic approaches*- the analysis of transgenic and gene-targeted mice that have modifications that interfere with or enhance PI3K activation has shed

considerable new light onto the role of PI3K. Table 1.3 summarises the different approaches employed and their outcome.

Target	Viability	Outcome/conclusion	Reference
<b>p110<math>\alpha</math></b>	Die <i>in utero</i>	Important in Cell growth/survival	(Bi et al., 1999)
<b>p110<math>\beta</math></b>	Die <i>in utero</i>	Important in Cell growth/survival	(Bi et al., 2002)
<b>Double heterozygous p110<math>\alpha</math>/p110<math>\beta</math></b>	Grow normally	Distinct roles in fetal development	(Bi et al., 2002)
<b>p110<math>\delta</math></b>	Grow normally		(Sasaki et al., 2000; Laffargue et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002)
<b>p110<math>\gamma</math></b>	Grow normally		(Sasaki et al., 2000; Laffargue et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002)
<b>p85 deficient mice expressing p55<math>\alpha</math> and p50<math>\alpha</math></b>	Viable		(Suzuki et al., 1999; Terauchi et al., 1999)
<b>p85<math>\alpha</math>/p55<math>\alpha</math>/p50<math>\alpha</math></b>	Die after birth	p55 $\alpha$ and p50 $\alpha$ have an essential role in vivo	(Fruman et al., 1999)
<b>p85<math>\beta</math></b>	viable	No apparent defects	(Ueki et al., 2002)
<b>SHIP</b>	Born-but have a shorter life span than wild-type		(Helgason et al., 1998; Liu et al., 1999)
<b>PTEN</b>	Embryonic lethal		(Di Cristofano et al., 1998; Suzuki et al., 1998)
<b>Class II</b>	Not yet generated		
<b>Class III</b>	Not yet generated		

**Table 1.3. Summaries of the different genetic approaches employed to identify the role of PI3K isoforms and their outcome (Koyasu, 2003)**

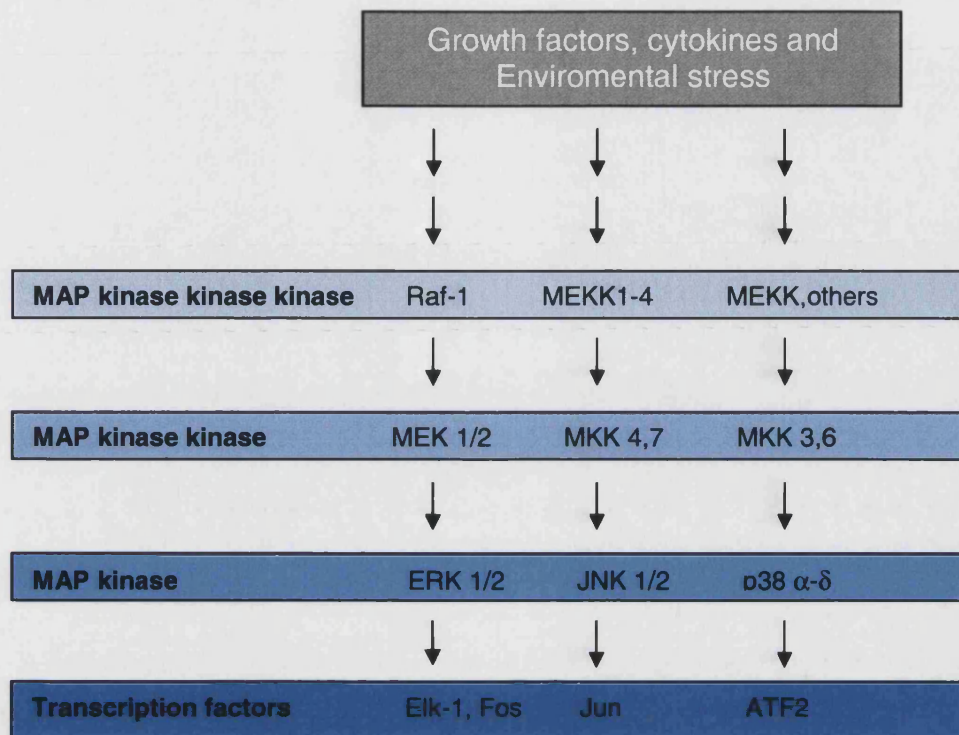


### **1.4.2. Mitogen-activated protein kinase (MAPK)**

The MAPKs are a major group of pathways which transduce extracellular signals to intracellular responses. All subfamilies participate in three tier protein phosphorylation cascades (shown in Figure 1.11) that serve to mediate diverse cellular processes in response to extracellular signals. The members of the MAPK pathways share core features concerning their constituent kinases:

1. All MAP kinases are activated via phosphorylation of both Thr and Tyr in a Thr-Xxx-Tyr motif.
2. This activation is catalysed by a dual specificity serine-threonine MAP kinase kinase (MAPKK) which, in turn, is phosphorylated and activated by an upstream MAP kinase kinase kinase (MAPKKK)
3. Once activated all MAP kinases can phosphorylate and activate other kinases or transcription factors, in the presence of ATP, to exert their biological effects.

There are three main categories of MAPK – extracellular-regulated protein kinase (ERK or p42/44), p38 MAP kinase and c-Jun NH2-terminal kinase (JNK or p46/54) – and these shall be considered in turn below.



**Figure 1.11. MAP kinase superfamily of serine/threonine kinases.** Each MAP kinase family member-ERK, JNK and p38- is activated by successive activation of a MAP kinase kinase and MAP kinase kinase.

### **ERK**

Extracellular-regulated protein kinase (ERK) was the initial classical MAPK and is activated in response to proliferation and differentiation factors, mediated by receptor tyrosine kinases, heterotrimeric G protein-coupled receptors, cytokine receptors and integrins. Receptor ligand interaction promotes the recruitment of the small guanosine triphosphatase (GTPase) Ras, which then activates the serine-threonine kinase raf, which can activate members of the MAPK /ERK kinase (MEK) family. ERK1 (p44) and ERK2 (p42) are activated by phosphorylation at residues



Thr<sup>202</sup> and Try<sup>204</sup> by MEK1 and MEK2 which are acting as MAPKKs. In this context raf is acting as a MAPKKK.

Downstream of ERK activation are its effectors, which include regulatory molecules, the transcription factors ATF2 and Elk, and the MAP kinase activated protein kinases (MAPKAPKs) 1 and 5. Thus, ERK2 is a crucial element for maintenance of cell homeostasis in response to changes in extracellular environment. Study of the cellular effects of ERKs has been made possible by the use of small specific MEK1/2 inhibitors such as PD90859 (Alessi et al., 1995).

### ***p38***

The p38 MAP kinase is often grouped with c-Jun NH2-terminal kinase (JNK) as the stress activated protein kinases. They are both involved in regulating the cells responses to stress, inflammation and also apoptosis pathways. The p38 MAP kinase isoforms ( $\alpha$ ,  $\beta$ ,  $\beta 2$ ,  $\gamma$  and  $\delta$ ) share a Thr-Gly-Tyr motif and are preferentially activated by pro-inflammatory cytokines (TNF $\alpha$  and IL-1), UV light, as well as heat and osmotic shock. There is some confusion over the precise details of the activating pathways for p38 MAP kinase which stems from the convergence of many upstream kinase dependent pathways (Herlaar and Brown, 1999; Ichijo, 1999). However p38 is activated by the MAPKKs, MKK3 and MKK6, which act as points of convergence for MAPKKK pathways.

The effects of p38 activation are mediated by activation of transcription factors such as ATF2 and Elk1 as well as activating MAPKAPKs 2, 3 and 5. The study of the

functional consequences of its activation is assisted by the use of specific inhibitors of the pyridinyl imidazole family, such as SB203580 (Young et al., 1997; Davies et al., 2000) and SB202190.

### ***JNK***

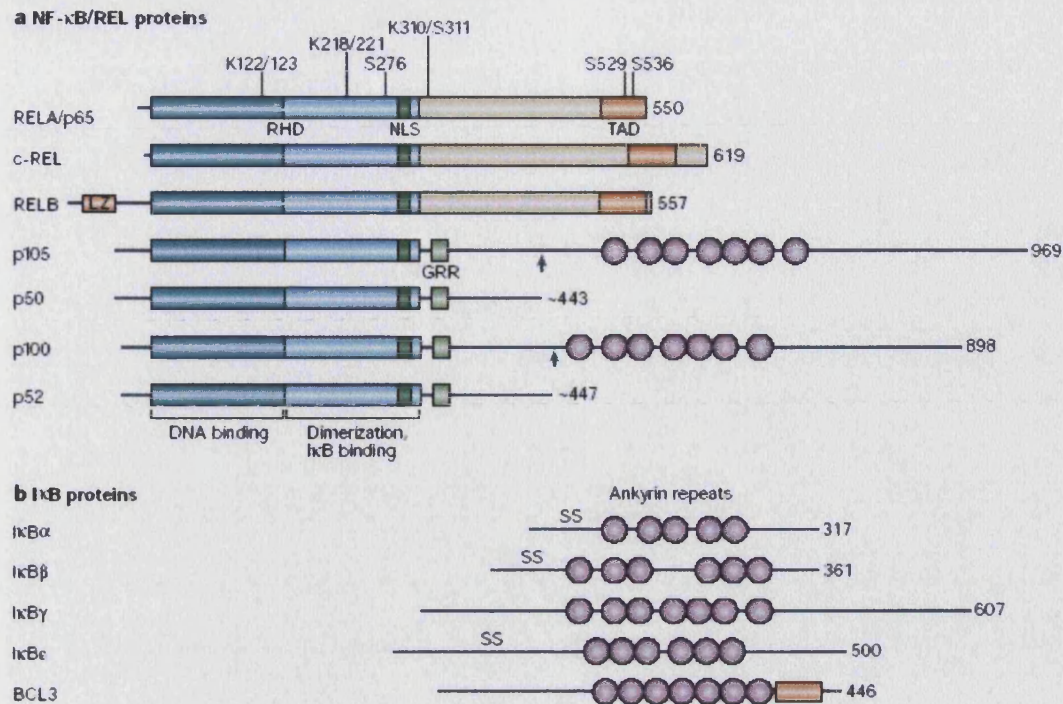
There are many similarities between p38 MAP kinase and JNK. They are both activated by similar cellular stresses and have similar functional consequences. JNKs are phosphorylated on their common Thr-Pro-Tyr motif by MKK4 and MKK7 which, as with p38, integrate a wide variety of triggering stimuli.

Downstream of JNK activation, and mediating its effects, is activation of the transcription factors AP1, ATF2 and Elk1. The functional consequences of this are believed to be similar to p38 activation. Again, the study of cellular effects of JNK have been made possible by the use of newly available JNK inhibitors such as SP600125- a reversible ATP-competitive inhibitor (Bennett et al., 2001). A cell-permeable, biologically active peptide consisting of a carboxyl terminal sequence derived from the JNK binding protein and an amino terminal peptide containing the HIV-TAT 48-57 sequence has also been developed. This peptide diminishes JNK signalling by blocking the activation of the transcription factor c-jun. This effect appears to be due to inhibition of phosphorylation of the activation domains of JNK (Bonny et al., 2001; Barr et al., 2002)

### **1.4.3. Transcription factors**

#### **Nuclear factor $\kappa$ B (NF $\kappa$ B)**

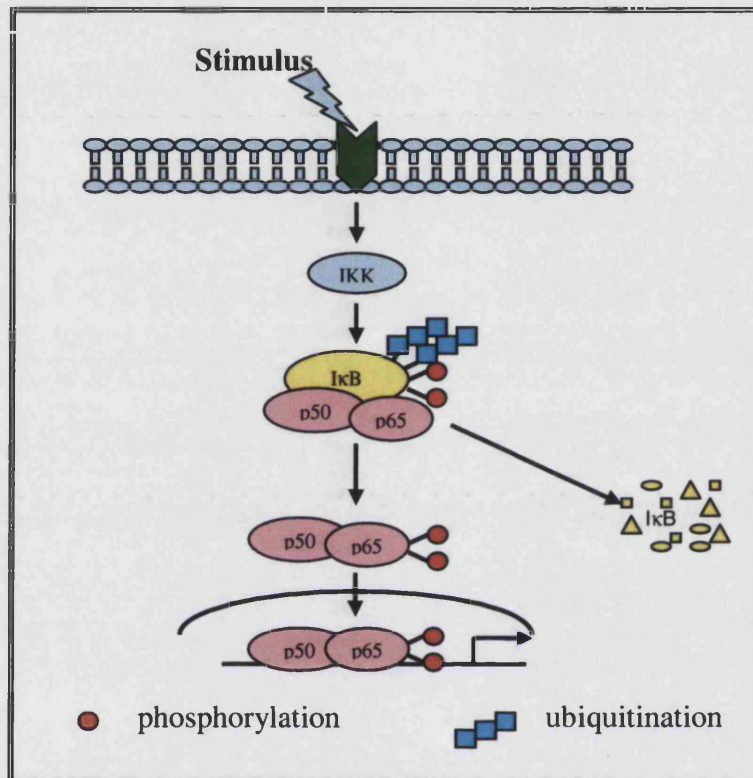
NF $\kappa$ B is an inducible transcription factor first described as a B cell-specific factor that binds to a 10bp motif in the Ig  $\kappa$  light chain intronic enhancer (Sen and Baltimore, 1986a; Sen and Baltimore, 1986b). NF $\kappa$ B is present in all cells in a resting state in the cytoplasm; only when it is activated and translocated to the nucleus is the usual sequence of events generated. Currently, NF $\kappa$ B consists of a family of Rel homology domain (RHD)-containing proteins: Rel A (p65), Rel B, c-Rel, p50 (NF $\kappa$ B1), and p52 (NF $\kappa$ B2) (Figure 1.12). NF $\kappa$ B1 and NF $\kappa$ B2 are synthesized as large precursors of 105 (p105) and 100kDa (p100) respectively. These are partially proteolysed by the proteasome (termed processing), to produce the active NF $\kappa$ B1 p50 and NF $\kappa$ B2 p52 subunits. RelA and c-Rel both have C-terminal transactivation domains in their RHDs and can strongly activate transcription from NF $\kappa$ B binding sites in target genes. RelB also contains a transactivation domain in its RHD and can function as an NF $\kappa$ B activator when complexed with p50 or p52. However, RelB/RelA heterodimeric complexes are inhibitory, since they cannot bind DNA (Marienfeld et al., 2003). NF $\kappa$ B1 p50 and NF $\kappa$ B2 p52 lack a transactivation domain, and can only promote transcription when heterodimerized with a transactivating Rel subunit (Li and Verma, 2002). Homodimers of p50 and p52 function as transcriptional repressors, but can stimulate transcription when bound to the I $\kappa$ B-like nuclear protein BCL-3 (Franzoso et al., 1992; Bours et al., 1993; Fujita et al., 1993).



**Figure 1.12. The family of mammalian NFκB/REL and IκB proteins (acquired from (Chen and Greene, 2004)).** The amino-terminal portion of the RHD is responsible for both backbone and sequence-specific contacts within the major groove of the κB enhancer. The carboxy-terminal portion of the RHD mediates dimerization with other NFκB/REL-family members and forms the site for physical docking to the IκBs (Baeuerle, 1998). The p105 and p100 proteins contain ankyrin repeats (indicated by pink circles), as well as glycine-rich regions (GRRs). The GRRs are important for co-translational processing of p105 to p50 (Lin et al., 1998) and post-translational processing of p100 to p52 (Senftleben et al., 2001; Xiao et al., 2001; Claudio et al., 2002; Coope et al., 2002). Phosphorylation of RELA at serines (S)276, S311, S529 and/or S536 is required for optimal NFκB transcriptional activity. Acetylation of RELA at lysines (K)122, K123, K218, K221 and K310 regulates distinct functions of NFκB, including DNA binding, IκBα association and RELA-mediated transactivation. The leucine zipper (LZ) of RELB is required for transactivation by RELB. NFκB action is linked to the biology of its inhibitory proteins, the IκBs (see part b of the figure). A hallmark of these IκB proteins is an ankyrin-repeat domain, which mediates the assembly with REL proteins. When bound by IκBα, the nuclear localization signal (NLS) of RELA is masked, and RELA cannot localize to the nucleus or bind to DNA. Phosphorylation of two serine residues (SS) at the amino-terminal region of IκBα triggers polyubiquitylation and proteasome-mediated degradation of IκBα (Chen and Greene, 2004).

In unstimulated cells, NFκB dimers are inactive, since they are sequestered in the cytoplasm by interaction with inhibitory proteins termed IκBs (inhibitors of NFκB) (Ghosh et al., 1998). In response to stimulation with agonists, IκBs are proteolytically degraded by the proteasome, releasing associated NFκB dimers to

translocate into the nucleus and modulate gene expression (Figure 1.13). The transcriptional activity of certain NF $\kappa$ B dimers is additionally regulated by phosphorylation, providing an extra level of control of NF $\kappa$ B activation (Chen and Greene, 2004).



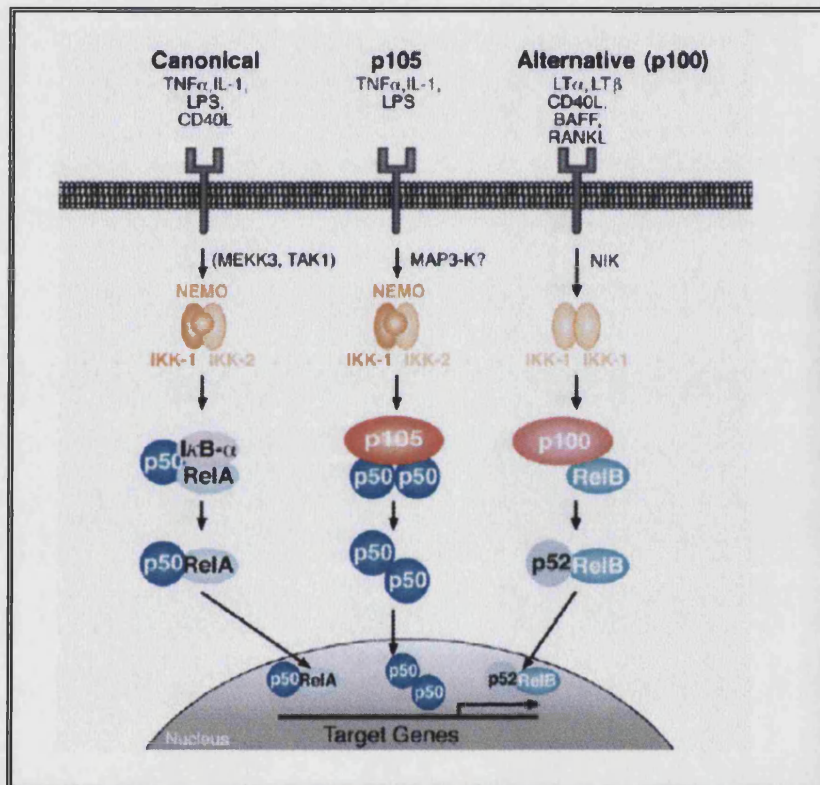
**Figure 1.13. Model of generic NF $\kappa$ B activation pathway.** Various stimuli induce the phosphorylation and subsequent polyubiquitination of I $\kappa$ Bs, which are then targeted for degradation. Associated NF $\kappa$ B dimers are thereby released to translocate into the nucleus, where they bind to the promoter regions of NF $\kappa$ B responsive genes to modulate their expression. The transactivating capacity of nuclear NF $\kappa$ B dimers can also be regulated by phosphorylation.

The I $\kappa$ Bs comprise a family of ankyrin-domain-containing proteins that keep NF $\kappa$ B in its inactive state within the cytoplasm by masking nuclear localization signal domains (Siebenlist et al., 1994; Miyamoto and Verma, 1995). This family includes I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\epsilon$ , bcl-3, p105 and p100 (Figure 1.12). I $\kappa$ B $\alpha$  interacts only weakly with p50 homodimers, and does not efficiently prevent their translocation to the nucleus (Siebenlist et al., 1994)



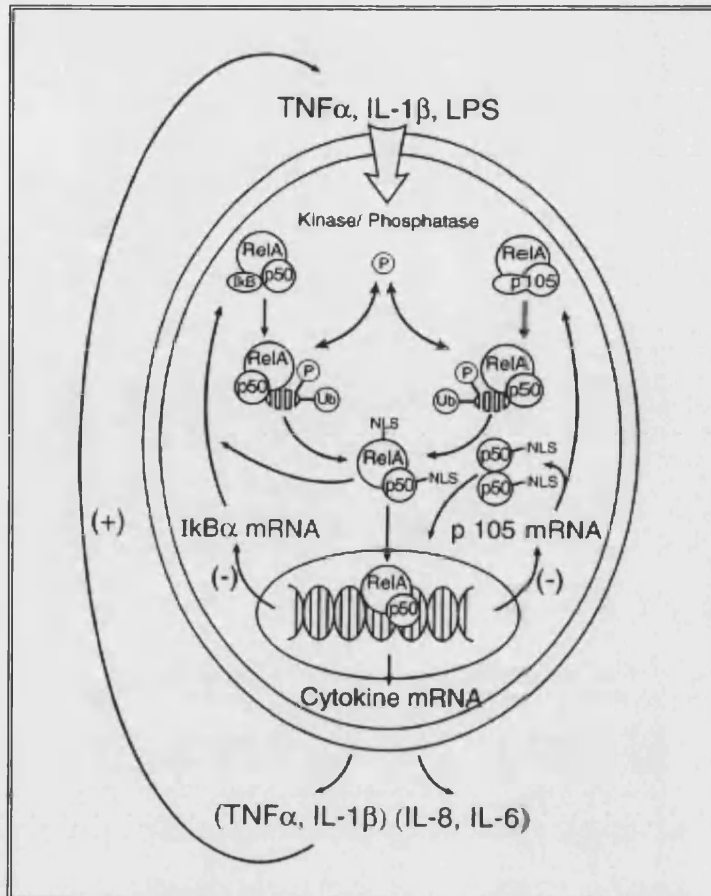
**Classical (canonical) NF $\kappa$ B pathway**

The prototype NF $\kappa$ B heterodimer is composed of the RelA (p65) and NF $\kappa$ B1 (p50) subunits. This combination, which is the component of the NF $\kappa$ B canonical (classical) pathway, is the most potent gene activator among the NF $\kappa$ B family (Figure 1.14).



**Figure 1.14 Three NF $\kappa$ B pathways.** The canonical pathway (left) is activated by a large number of agonists, a few of which are listed. Activation of this pathway depends on the IKK complex (IKK1 IKK2–NEMO), which phosphorylates I $\kappa$ B $\alpha$  to induce its rapid degradation. (I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$  are similarly regulated by IKK.) This pathway is essential for immune responses, inflammation and promoting cell survival. The alternative NF $\kappa$ B pathway (right) is activated a limited number of agonists and is important for secondary lymphoid organogenesis, maturation of B-cells and adaptive humoral immunity. This pathway requires NIK and IKK1 and induces the slow processing of p100 to p52, resulting in nuclear translocation of p52/RelB heterodimers. The p105 pathway (centre) is specifically involved in immune and inflammatory responses. Agonist activation of this pathway induces phosphorylation of the p105 PEST region by the classical IKK complex. This triggers p105 polyubiquitination and subsequent degradation, releasing p50 homodimers which undergo nuclear translocation and positively or negatively regulate gene transcription (Beinke and Ley, 2004).

The prototypical I $\kappa$ B protein is I $\kappa$ B $\alpha$ , which binds to the p65 subunit of NF $\kappa$ B in the cytoplasm of unstimulated cells keeping it in an inactive state. Pro-inflammatory cytokines such as TNF $\alpha$  triggers the activation of a complex of IKK, which includes the scaffold protein NF $\kappa$ B essential modulator (NEMO; also named IKK $\gamma$ ) (Yamaoka et al., 1998), IKK $\alpha$  and IKK $\beta$  kinases (Zandi et al., 1997). Once activated, the IKK complex phosphorylates the Ser<sup>32</sup> and Ser<sup>36</sup> residues of I $\kappa$ B $\alpha$ . This step initiates the degradation, by ubiquitination, of I $\kappa$ B $\alpha$  which allows the release of free NF $\kappa$ B. This reveals its nuclear translocation signal resulting in transportation to the nucleus and NF $\kappa$ B dependent gene transcription. Among the many genes with a NF $\kappa$ B response element is the gene for I $\kappa$ B $\alpha$  and p105 which is subsequently resynthesised (Cogswell et al., 1993; Ito et al., 1994) after activation. Increased production of inhibitory units presumably helps trap NF $\kappa$ B in the cytoplasmic compartment, and downregulates activated NF $\kappa$ B, thus terminating new cytokine transcription and limiting the inflammatory response by negative feedback. An interesting effect of increased production of p105 is that p50 homodimer formation is also increased, which may diminish NF $\kappa$ B-mediated responses to subsequent stimuli. Since p50 homodimers do not bind efficiently to I $\kappa$ B, and lack transcription-activation domains, they can translocate to the nucleus and function as inhibitors of NF $\kappa$ B mediated gene expression by competing with other Rel proteins for access to NF $\kappa$ B binding sites.



**Figure 1.15. Regulation of NFκB (RelA/p50) activation following cell stimulation with TNF-α, IL-1β, or endotoxin (LPS).** Binding of these agents at the cell surface signals phosphorylation and addition of ubiquitin (Ub) to the inhibitory unit (IκB-α or p105). These events lead to recognition and proteolysis of the inhibitor, exposing the nuclear localization signal (NLS) on p50 and RelA. RelA/p50 heterodimers bind to specific motifs in the promoter regions of cytokine genes, and initiate transcription. Cytokine mRNA is translated into proteins, which are secreted by cells. TNF-α and IL-1β can act locally to amplify NFκB activation. Nuclear binding of NFκB also stimulates production of IκB-α and p105, which inhibit further activation of NFκB. Increased p105 production favors the formation of p50 homodimers, which can inhibit NFκB action by entering the nucleus and competing for NFκB binding sites (Blackwell and Christman, 1997).

Positive feedback may occur through extracellular mechanisms that serve to amplify inflammatory signals. NFκB activation enhances the transcription of TNFα and IL-1β, and both of these cytokines are in turn known to activate NFκB. Both negative and positive feedback control of NFκB is outlined in Figure 1.15.

All members of the IκB family do not follow the same paradigm as IκBα. IκBβ is not regulated by NFκB and does not have this intrinsic negative feedback resulting



in persisting activation. Furthermore there is evidence of post-translational modification of RelA phosphorylation state providing an alternative mechanism of regulating NF $\kappa$ B (Egan et al., 1999).

### ***Alternative NF $\kappa$ B pathway***

In contrast to this canonical pathway which is activated by multiple stimuli, an alternative NF $\kappa$ B activation pathway has been described and is NEMO-independent (Figure 1.14). This pathway is activated by B cell-activating factor receptor, lymphotoxin- $\beta$  receptor, CD40, and latent membrane protein-1 of the Epstein-Barr virus (Cao et al., 2001; Dejardin et al., 2002). This signalling pathway relies on the recruitment of TRAF proteins to the membrane and on the NF $\kappa$ B-inducing kinase (NIK) (Xiao et al., 2001), which activates an IKK $\alpha$  homodimer-the substrate of which is p100. Once phosphorylated p100 is ubiquitinated and cleaved to generate the NF $\kappa$ B protein p52, which moves as a heterodimer with RelB into the nucleus.

### ***p105 NF $\kappa$ B pathway***

Cellular levels of p105 are regulated by two distinct proteolytic mechanisms which are mediated by the proteasome. Processing of p105 to generate p50 occurs constitutively, resulting in similar levels of p105 and p50 in most cell types (Karin and Ben Neriah, 2000). By contrast, agonist stimulation predominantly triggers complete degradation of p105, which releases associated Rel subunits to translocate into the nucleus. p105 can retain RelA, c-Rel and p50, but not RelB, in the cytoplasm (Rice et al., 1992; Mercurio et al., 1993; Solan et al., 2002). However,

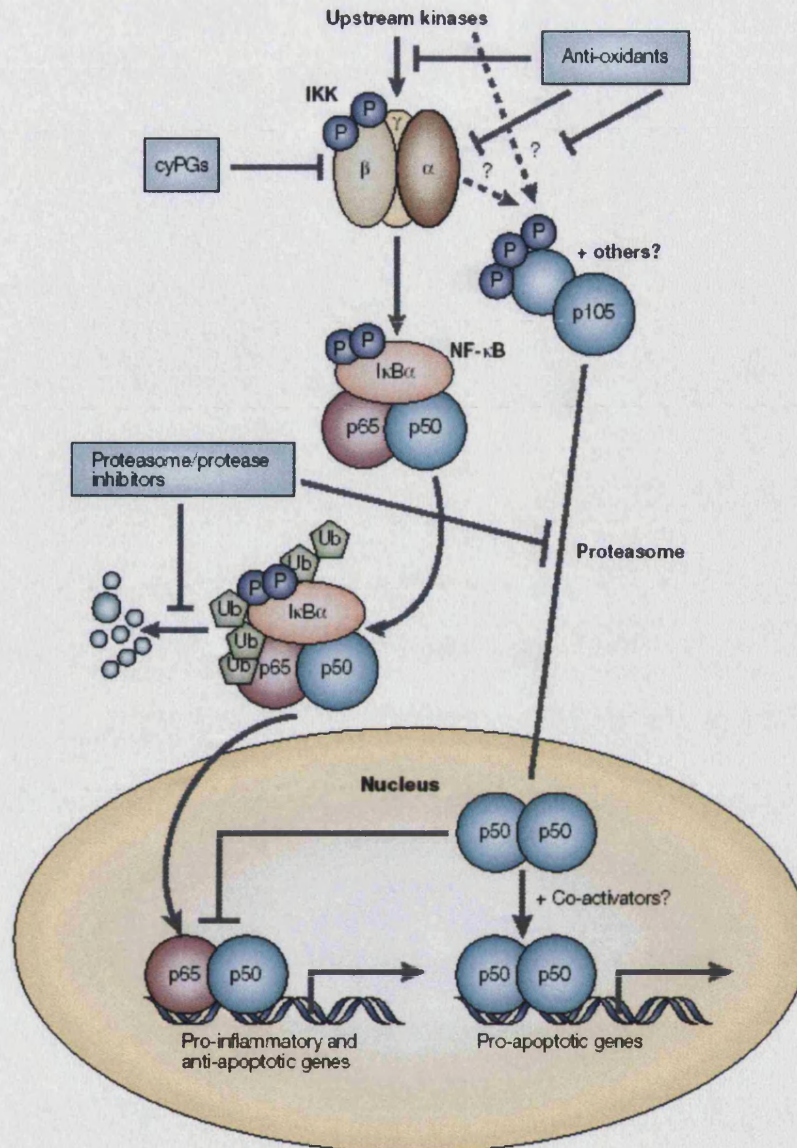
genetic studies in mice suggest that p105 is only essential for cytoplasmic retention of p50 (Ishikawa et al., 1998). It is unclear whether p105 binds directly to p50 homodimers or p50 monomers which subsequently dimerize upon release. I $\kappa$ B $\alpha$  binds inefficiently to p50 homodimers (Liou et al., 1992), but can bind to p50, which is heterodimerized with other Rel subunits, such as RelA (Figure 1.14).

### ***Regulation of genes by NF $\kappa$ B pathway***

Activated NF $\kappa$ B transcriptionally regulates a large number of genes including many immediate early (IE) genes. These include iNOS, IL-8, RANTES, TNF $\alpha$ , IL-1 $\beta$ , IL-6, ICAM-1 and COX-2. With regard to COX-2, its induction has been shown to be NF $\kappa$ B dependent in A549 lung epithelial cells (Newton et al., 1997). The genes which are transcriptionally activated by NF $\kappa$ B are generally pro-inflammatory and include cytokines that activate NF $\kappa$ B allowing potential amplification of the inflammatory response that needs to be tightly regulated. Furthermore, NF $\kappa$ B often functions in cooperation with other transcription factors, such as AP-1 and C/EBP, that are also involved in regulation of inflammatory and immune genes (Stein and Baldwin, Jr., 1993; Stein et al., 1993)

### ***Role of NF $\kappa$ B in the resolution of inflammation?***

In contrast to the well-documented pro-inflammatory role of NF $\kappa$ B, Lawrence et al., (2001) described an active role for NF $\kappa$ B in the resolution of inflammation (Figure 1.16).



**Figure 1.16. Hypothetical model of an alternative anti-inflammatory pathway of NFκB activation.** Broad-spectrum nuclear factor-κB (NFκB) inhibitors that target the proteasome or undefined upstream signalling events could block potentially beneficial anti-inflammatory pathways of NFκB activation (Lawrence et al., 2002). For example, they might block the processing of p105 to p50 and the assembly of p50–p50 homodimer complexes, which negatively regulate pro-inflammatory gene expression and might coordinate the expression of an alternative set of NFκB target genes through interaction with distinct co-activators. The specificity of cyclopentenone prostaglandins (cyPGs) for inhibitor of NFκB kinase-β (IKKβ) might prevent undesirable side effects of inhibitors through the inhibition of other IKKs or IKK-independent pathways for NFκB activation. Anti-oxidants have been shown to inhibit many stages of the NFκB pathway, including the activation of upstream IKK kinases and IκB kinase activity. P, phosphate; Ub, ubiquitin.

This involved the recruitment of alternative DNA-binding complexes that lack transactivation domains, such as p50–p50 homodimers. Broad-spectrum inhibitors of

the NF $\kappa$ B pathway, such as antioxidants and proteasome inhibitors, had the expected anti-inflammatory actions during the initiation of inflammation; however, when they were administered after the onset of inflammation, these inhibitors prevented the resolution of inflammation, which was associated with the inhibition of leukocyte apoptosis. The authors hypothesize that this alternative NF $\kappa$ B pathway promotes leukocyte apoptosis by the transactivation of pro-apoptotic target genes with co-activating factors. Alternatively, active NF $\kappa$ B DNA binding complexes that lack transactivation domains might act as dominant-negative inhibitors of anti-apoptotic gene expression (Figure 1.16). CyPGs might repress the activation of IKK $\beta$ , leading to a predominance of anti-inflammatory signalling pathways that are regulated independently of IKK or by an alternative IKK complex that is not sensitive to inhibition by cyPGs (Lawrence et al., 2002)

### Activating protein-1 (AP-1)

AP-1 is a heterodimer of Fos and Jun, and is a member of the basic leucine zipper (bZIP) transcription family, characterized by a basic leucine-rich area that is involved with dimerization with other transcription factors (Figure 1.17).

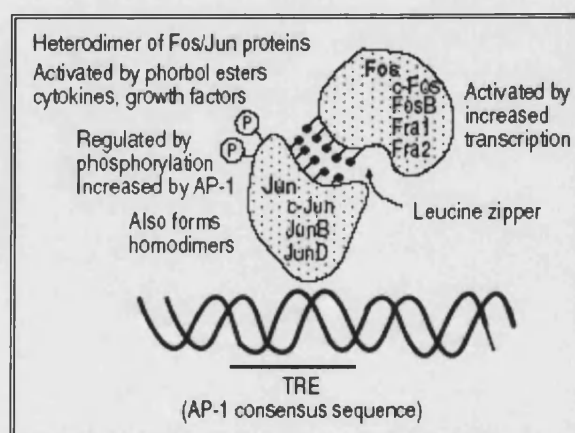


Figure 1.17. Activator protein (AP)-1 is a heterodimer of Fos and Jun proteins

AP-1 is a collection of related transcription factors belonging to the Fos (cFos, FosB, Fra1, Fra2) and Jun (c-Jun, JunB, JunD) families that dimerize in various combinations through their leucine zipper region. Fos/Jun heterodimers bind with the greatest affinity and are the predominant form of AP-1 in most cells, whereas Jun/Jun homodimers bind with a low affinity.

AP-1 may be activated via PKC and by various cytokines, including  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$  via several types of protein tyrosine kinase and MAPK, which themselves activate a cascade of intracellular kinases (Karin, 1995; Karin et al., 1997). Both  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$  activate TRAF, which subsequently activates MAP kinases (Eder, 1997). Recent studies suggest that there may be interactions between the AP-1 activating pathway and  $\text{NF}\kappa\text{B}$  pathways (Eder, 1997).

AP-1 activation in human lung after stimulation with PMA,  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$  has been demonstrated (Adcock et al., 1994a; Adcock et al., 1994b). There is evidence for increased expression of c-Fos in epithelial cells in asthmatic airways (Demoly et al., 1992), and many of the stimuli relevant to asthma that activate  $\text{NF}\kappa\text{B}$  will also activate AP-1. AP-1 like  $\text{NF}\kappa\text{B}$ , regulates many of the inflammatory and immune genes that are overexpressed in asthma. Indeed, many of these genes require the simultaneous activation of both the transcription factors that work together cooperatively.

#### 1.4.4. Signalling pathways downstream of cytokine receptors

Many signalling pathways are activated downstream of cytokine receptors. Although the cytokines TNF $\alpha$  and IL-1 $\beta$  have been introduced in terms of their biology, this next section describes TNF $\alpha$  and IL-1 $\beta$  receptors and how they couple to the signalling pathways described above.

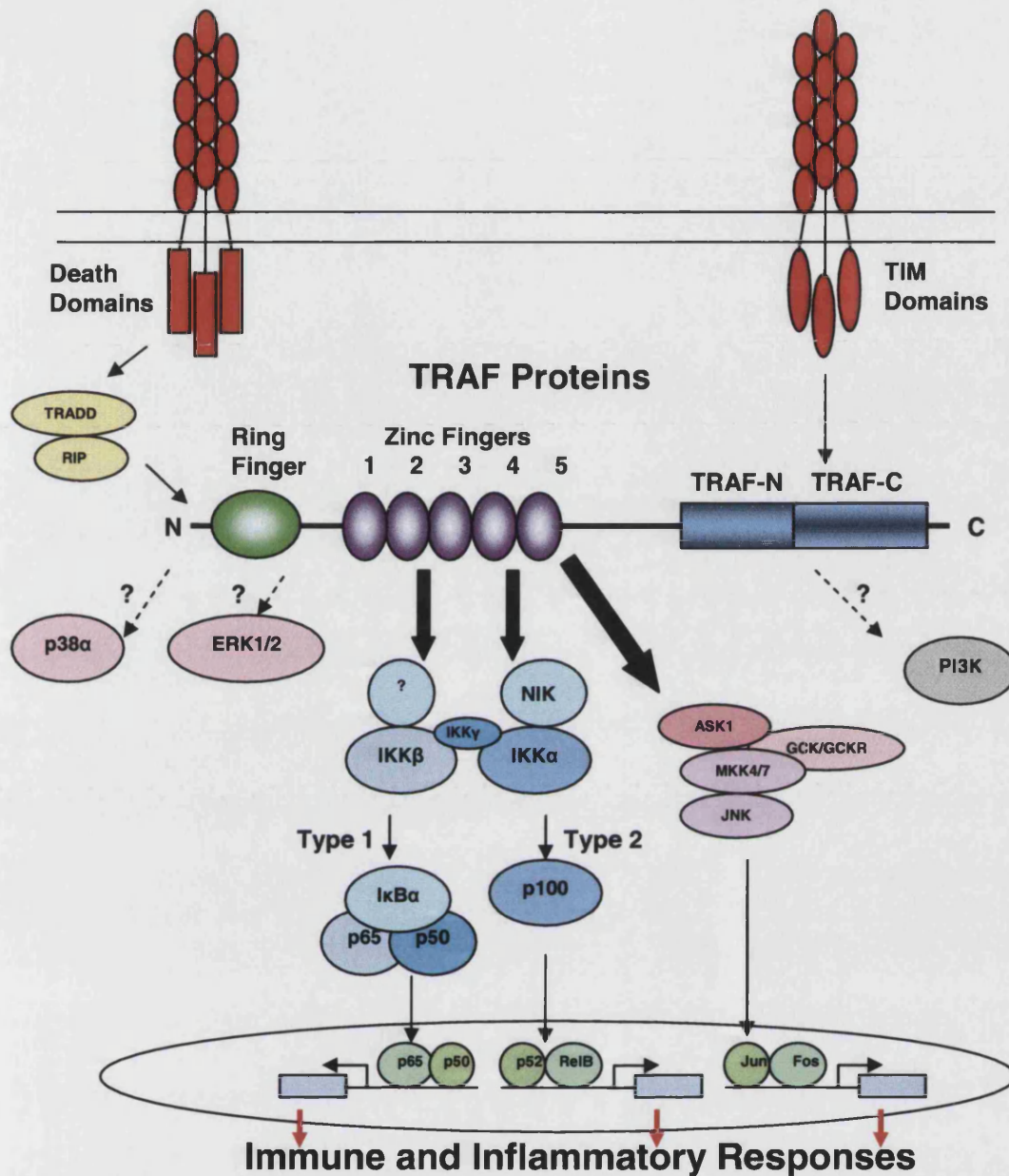
##### *TNF $\alpha$ receptors and signalling*

There are two distinct TNF $\alpha$  receptors which are also part of a larger family of related receptors. These receptors, TNFR1 (p55 or CD120a) and TNFR2 (p75 or CD120b), are expressed simultaneously on most cell types and it is therefore difficult to assign distinct signalling functions to each receptor (Narumiya and Fukushima, 1986; Orlinick and Chao, 1998). However experiments using *TNF $\alpha$* <sup>-/-</sup> and *TNFR1*<sup>-/-</sup> knockout mice have similar phenotypes with markedly reduced sensitivity to LPS after D-galactosamine priming (Pfeffer et al., 1993; Pasparakis et al., 1996). Furthermore the *TNF $\alpha$* <sup>-/-</sup> phenotype can be restored to that of wild type mice by the preparation of a transgenic mouse with human TNF $\alpha$  which can only bind TNFR (Pasparakis et al., 1996). Such studies indicate a central role for TNFR1 in TNF $\alpha$  signaling. The role of TNFR2 appears to be more complex. Evidence shows that the intracellular signals that couple TNFR1 include an intracellular signal protein termed TNF receptor-associated death domain (Hsu et al., 1995). Upon engagement of TNFR1, this intracellular signal protein acts as an adapter by recruiting the downstream transducer TNF-receptor associated factor (TRAF)-2, which stimulates NF $\kappa$ B (Hsu et al., 1995; Hsu et al., 1996).

Receptor-mediated multimerization of TRAFs has been found to activate multiple signalling pathways (Figure 1.18). The best described of these are the JNK and NF $\kappa$ B pathways which play a major role in the biological functions elicited by TNFR family members. In addition to these two pathways, reports have also documented TRAF-mediated activation of the ERK, p38 and PI3K pathways.

The TNF $\alpha$  promoter itself contains NF $\kappa$ B and AP-1 binding sites and is subject to positive autoregulation, a property that is important for the amplification of inflammatory responses. Once in the nucleus, NF $\kappa$ B transcriptional activity can be modulated further through phosphorylation by various protein kinases that are TNF $\alpha$  responsive, such as the p38 subgroup, providing a point for cross-talk with other signalling pathways. AP-1 is a heterogeneous collection of dimeric transcription factors comprising Jun, Fos and ATF subunits (as outlined above), whose activity is regulated by multiple mechanisms, including phosphorylation by various MAPK (Karin et al., 1997). JNKs are particularly relevant to TNF $\alpha$ -mediated induction of AP-1 activity, although the p38 MAPKs also affect AP-1 activity ( Han et al., 1997; Karin et al., 1997)





**Figure 1.18 Signalling pathways activated by TRAFs.** This model depicts a partial view of the known pathways activated by TRAF proteins. TRAFs activate the JNK, p38, ERK, PI3K and the types 1 (classical) and 2 (atypical) NFκB pathways. TRAF-mediated activation of the JNK and NFκB pathways has been studied extensively and these pathways are depicted in detail (Dempsey et al., 2003).

### *IL-1 receptors and signalling*

The IL-1R1 is the IL-1 receptor mediating functional responses and shares a 45% homology with the *Drosophila Toll* gene. When the membrane bound form binds to IL-1α or β it associates with the IL-1RAcP to form a signalling complex which lacks any intrinsic kinase activity. It does however have a binding site at amino acids 513-



520 for IL-1R1 associated kinase (IRAK) which can phosphorylate a 100kDa substrate and is partly responsible for downstream activation of NFκB, MAP kinases, and activation of the AP-1 transcription factor. However there is also evidence that IL-1 can activate PI3K and, as with TNFα, activation of NFκB may be PI3K dependent (Reddy et al., 2000).

#### **1.4.5. Role of signalling pathways in respiratory diseases**

##### ***PI3K and asthma***

Although reversible airway narrowing leading to wheeze, cough and shortness of breath are hallmark features of asthma, patients with longstanding and severe disease can develop fixed airways obstruction that is associated with structural changes within the airway wall. The most prominent feature of the remodelling airway is an increase in airway smooth muscle (ASM). It is clear from a number of diverse approaches that the PI3K pathway plays an important role in regulating progression cell cycle control and thus airway remodelling (Walker et al., 1998; Krymskaya et al., 1999). In addition, p70<sup>S6K</sup>, a downstream target of PI3K/PKB, has been shown to be an important mediator in human ASM cell mitogenesis (Krymskaya et al., 1999).

PI3K is thought to contribute to the pathogenesis of asthma by effecting the recruitment, activation and apoptosis of inflammatory cells. The role of Class IA PI3K in antigen-induced airway inflammation and hyperresponsiveness has been investigated by i.p. administration of Δp85 protein, a dominant negative form of

p85 $\alpha$ , fused to HIV-TAT (TAT- $\Delta$ p85) into mice (Myou et al., 2003). These authors showed that PI3K has a regulatory role in Th2-cell cytokine secretion, airway inflammation and airway hyperresponsiveness in mice (Myou et al., 2003).

### ***PI3K and neutrophils***

Neutrophils are recruited to the lungs in many respiratory disease states including fatal asthma. They cause tissue damage by their capacity to release toxic oxygen radicals (generated by the NADPH oxidase complex), the exocytosis of granules containing highly histotoxic compounds such as elastase and collagenases, and the elaboration and release of additional pro-inflammatory cytokines. PI3K have been shown to be key regulators of both neutrophil recruitment and activation. In mice lacking the catalytic subunit of the myeloid restricted PI3K $\gamma$ , neutrophil migration to the inflamed peritoneum was severely compromised (Hirsch et al., 2000; Sasaki et al., 2000). The accumulation of PtdIns(3,4,5)P<sub>3</sub> also correlated precisely with respiratory burst activity, in that neutrophil priming by agents such as TNF $\alpha$  markedly enhanced both the size and the duration of the release of superoxide anions and the accumulation of PtdIns(3,4,5)P<sub>3</sub> (Condliffe et al., 1998). In these cells PI3K inhibitors abolish the production of oxygen radicals induced by physiological agonists; neutrophils from PI3K $\gamma$  knockout mice exhibit a diminished respiratory burst, with residual activity most probably attributed to the action of remaining Class IA PI3K.

### ***PI3K and eosinophils***

Eosinophils accumulate in the bronchial tree and lung parenchyma in a number of disease states including asthma. Toxic eosinophil-derived mediators such as eosinophil cationic protein, major basic protein and oxygen radicals can damage the airway epithelium and are thought to contribute significantly to airway hyperresponsiveness. The migration of eosinophils to a number of chemoattractants seems to be sensitive to wortmannin (Dunzendorfer et al., 1998). The role of PI3K in eosinophil degranulation is not known, but these enzymes are required for activation of the eosinophil NADPH oxidase complex (Hofmann et al., 2000).

### ***PI3K and macrophages***

Alveolar macrophages undertake a number of key host defence functions within the lung. These include the phagocytosis of inhaled particles and respiratory pathogens, antigen presentation, and the generation of inflammatory cytokines. Additionally, they may be important in the resolution of acute inflammation by the ingestion of apoptotic neutrophils. So far, although few studies have addressed the role of PI3K in the alveolar macrophage, such data are available for monocyte-derived macrophages, macrophage cell lines and murine peritoneal macrophages. Murine PI3K $\gamma$ -null macrophages show decreased migration towards a variety of chemotactic agents, and greatly diminished recruitment to the inflamed peritoneum (Hirsch et al., 2000). The induction of cytokine gene expression in monocytes stimulated by formylated peptide has also been shown to be sensitive to PI3K inhibition (Pan et al., 2000). More importantly, the consequence of excessive PI3K activation has also been explored; mice deficient in SHIP suffer from lethal infiltration of the lungs by

myeloid cells, principally macrophages (Helgason et al., 1998). As with neutrophils and eosinophils, it seems that one or more of the PI3Ks is required for macrophage recruitment to the lung and for at least a subset of activation responses.

### ***PI3K and epithelial cells***

Although comparatively little is known about the function of the PI3Ks in other pulmonary cells, several reports have indicated the global importance of this signalling pathway in other settings in the lung. For example, PI3K has been reported to mediate lung epithelial cell differentiation and surfactant protein expression in fibroblasts induced by growth factor-2 (Matsui et al., 1999), although subsequent reports have suggested that PI3K inhibits surfactant secretion from type II alveolar cells (White and Strayer, 2000). The activation of PI3K by the pro-inflammatory Th1 cytokine TNF $\alpha$  has been shown in a number of cell systems, including epithelial cells (Hanna et al., 1999; Pastorino et al., 1999).

In apparent conflict with the pro-inflammatory role of PI3K in inflammation outlined previously, is the activation of PI3K in intestinal epithelial cells by the Th2 cytokine IL-13 which in some settings is thought to be regarded as being anti-inflammatory (Wright et al., 1997). This is supported by work in other systems demonstrating the activation of PI3K by IL-13 and other Th2 cytokines including IL-4 and IL-10 (Mirmonsef et al., 1999; Ceponis et al., 2000). In the intestinal epithelium the activation of PI3K by IL-13 was shown to be the mechanism mediating the inhibition of iNOS by this cytokine (Wright et al., 1997).

### ***MAPK and asthma***

The ERK pathway in addition to the PI3K pathway appear to be key positive regulators of airway smooth muscle mitogenesis. Bronchoalveolar lavage fluid from asthmatic airways has been shown to increase ERK activation (Naureckas et al., 1999). Cui et al. (2002), suggested the role of both ERK1/2 and p38 MAP kinase in eotaxin-induced cytokine production in bronchial epithelial cells. Thus these observations suggest the MAPK pathway plays a role in asthma.

Overstretching the airways during positive pressure mechanical ventilation or attacks of acute severe asthma are associated with important biologic responses (Oudin and Pugin, 2002). These authors showed that the MAPK pathway, and p38 in particular, are proximal and key intracellular signaling molecules mediating cell activation in response to cyclic stretch, a mechanical strain similar to that applied to lung epithelial cells during mechanical ventilation.

Since the MAPK pathways play a crucial role in the activation of transcription factors it is not surprising that these pathways therefore are important in the induction of pro-inflammatory mediators in respiratory diseases. The MAPK cascade is one of the most important signalling pathways involved in expression of COX-2 gene expression and PG biosynthesis, and contributes to the induction of COX-2 gene concertedly or independently (Su and Karin, 1996). Once activated, MAPKs can phosphorylate and activate transcription factors which regulate gene expression. IL-1 $\beta$  rapidly activates JNK/SAPK and p38 MAPK and also induces COX-2 expression (Guan et al., 1998). The COX-2 gene is an important Ras target.

Furthermore, studies suggest induction of COX-2 by some mitogens requires activation of Ras/Rac1/MEKK1/JNKkinase/JNK and/or the activation of Ras/Raf1/MAPKK/ERK signal transduction pathway.

The ERK pathway has also been shown to be of particular importance in the induction of cyclin D1, IL-1 $\beta$ , IL-5R, IL-6, IL-8, eotaxin, and RANTES. In addition, the p38 pathway has been shown to be involved in the induction of IL-6, IL-8, eotaxin, and RANTES (Gerthoffer and Singer, 2003).

### **1.5. Peroxisome proliferators activated receptors**

PPAR $\gamma$  ligands have been shown to modulate inflammation via regulation of COX-2. Furthermore the COX-2 promoter is thought to have a peroxisome proliferator response element (PPRE) (Meade et al., 1999). This next section describes PPARs and in particular PPAR $\gamma$  and gives an overview of how this receptor is thought to regulate the inflammation and the signalling mechanisms currently known to be involved.

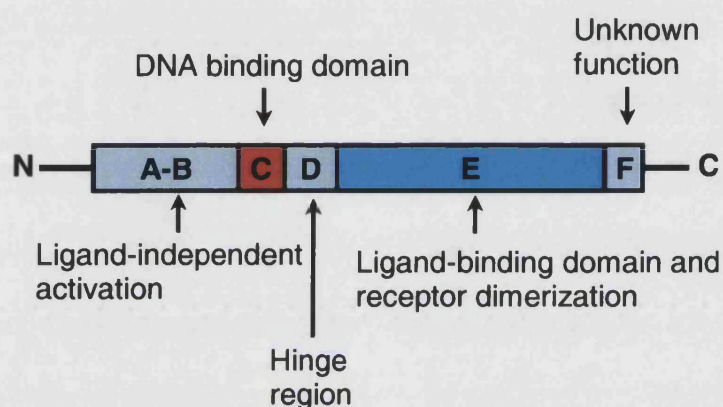
#### **1.5.1. Background**

As previously mentioned there are three isoforms of PPAR- PPAR $\alpha$ , PPAR $\delta$  (previously known as PPAR $\beta$ ) and PPAR $\gamma$ . The PPAR $\gamma$ 1 and PPAR $\gamma$ 2 subisoforms differ only in that  $\gamma$ 2 has 30 additional amino acids at its N terminus due to differential promoter usage within the same gene and subsequent alternative RNA

processing. All isoforms have been cloned and characterized by their distinct expression pattern, different ligand binding specificity and metabolic functions.

### Structure

PPARs have a similar structural organization to other nuclear hormone receptors (Figure 1.19).

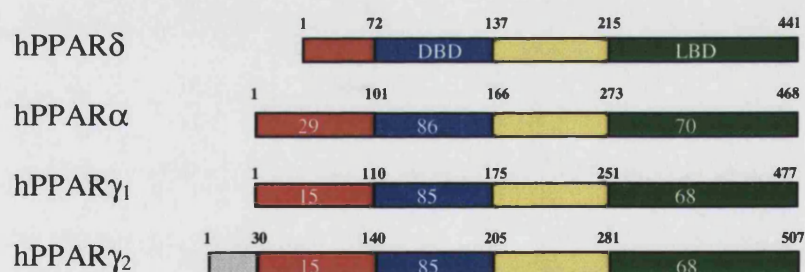


**Figure 1.19. Structure of nuclear hormone receptors.**

The N-terminal A/B domain contains a ligand independent trans-activation domain, termed activation function 1 (AF-1). This domain plays an important role in regulating PPAR activity through both phosphorylation by MAPK and interdomain communication (Hu et al., 1996; Shao et al., 1998; Juge-Aubry et al., 1999). This region is followed by the C domain which encodes the DNA binding domain that includes two zinc fingers. The C domain is responsible for the binding of PPAR receptor to PPRE in the promoter region of target genes. The D hinge region, which follows the C domain, is a docking domain for cofactors (Guan and Breyer, 2001). At the carboxyl terminus is a dimerization and ligand binding domain, which has a key ligand dependent trans-activation function called AF-2 (Nolte et al., 1998a;

Guan and Breyer, 2001), critical for both ligand-binding and recruitment of PPAR cofactors.

PPAR isoforms share a high degree of amino acid sequence similarity, both within their DNA- and ligand-binding domains (Kliewer et al., 1994) (Figure 1.20). This is reflected in functional similarities in that the receptors are activated by structurally related compounds and bind as heterodimers with a retinoid X receptor partner to a PPARE. Nevertheless, the three PPAR subtypes appear to serve different roles *in vivo*. They exhibit markedly different tissue distributions (Jones et al., 1995; Braissant et al., 1996), have differing affinities for different PPAREs (Brun et al., 1996), and are activated to different extents by different activators/ligands (Kliewer et al., 1994; Forman et al., 1995; Yu et al., 1995; Brun et al., 1996) which will be outlined later.

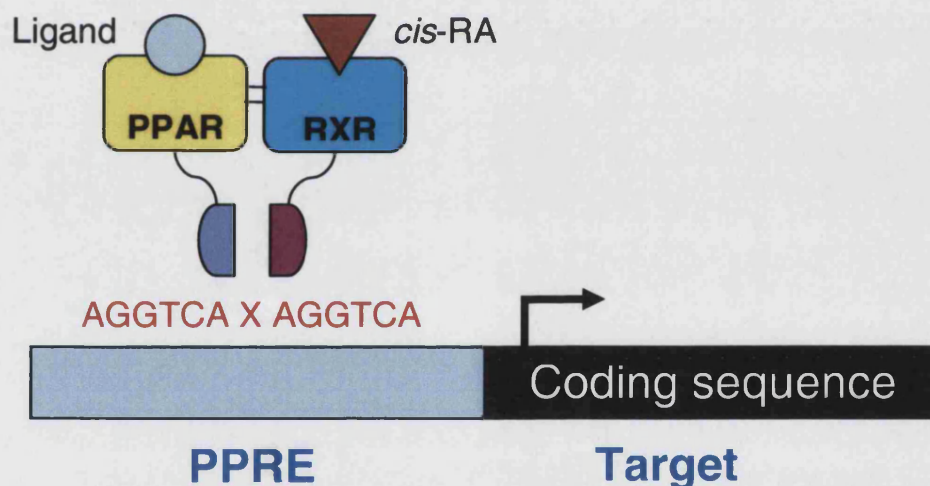


**Figure 1.20. PPAR isoforms share a common domain structure.** Human PPAR $\delta$ , PPAR $\alpha$ , and PPAR $\gamma$  are represented in linear fashion to display a conserved domain structure with a DNA binding domain (DBD) and ligand binding domain (LBD). Amino acid numbers are above each receptor, whereas percent identity at the amino acid level is displayed within each domain. PPAR $\gamma_1$  and PPAR $\gamma_2$  are distinguished by 30 extra amino acids at the N terminus of PPAR $\gamma_2$  (adapted from (Rosen and Spiegelman, 2001))



**Heterodimerization**

PPARs regulate gene expression by forming a heterodimer with a retinoid X receptor (RXR). RXRs are members of the nuclear hormone superfamily that are activated following the binding of 9-cis retinoic acid (RA) (Murphy and Holder, 2000). Like PPARs, RXR subfamily consists of three isoforms, RXR $\alpha$ , RXR $\beta$  and RXR $\gamma$  (Mangelsdorf et al., 1992). It is not known whether any one of the RXR isoforms preferentially binds one or more of the PPAR isoforms (Clark, 2002). However synthetic RXR agonists can activate the complex and thereby obtain antidiabetic outcomes similar to those seen with PPAR agonists in mouse models of type 2 diabetes (Mukherjee et al., 1997).



**Figure 1.21. Heterodimerization of PPAR and binding to PPARE**

Following ligand binding to PPAR, the conformation of PPAR is altered and stabilized such that a binding cleft is created and heterodimerization occurs (Berger and Moller, 2002), the PPAR:RXR heterodimer binds to specific response elements (PPREs) in the promoter regions of target genes. PPREs contain one or more copies

of the hexameric DNA consensus AGGTCA arranged as a direct repeat spaced by one nucleotide. Such a sequence, or similar one, has been found in numerous PPAR-inducible genes including acyl-CoA oxidase and adipocyte fatty acid-binding proteins (Wahli et al., 1995). Binding of the heterodimer to the PPRE can thus regulate gene expression (Figure 1.21).

### ***Co-activators and co-repressors***

PPAR-regulated transcriptional activation is made more complex by the involvement of co-activators and co-repressors. Several cofactor proteins, coactivator, and corepressors that mediate the ability of nuclear receptors to initiate (or suppress) the transcription process were recently identified (Xu et al., 1999b). High-affinity complexes are formed between the PPAR-RXR heterodimer and nuclear receptor co-repressor protein, in the absence of ligand (Murphy and Holder, 2000). These prevent transcriptional activation by sequestering the nuclear receptor heterodimer from the promoter. As a consequence of the conformational change induced by binding of ligand, the co-repressors dissociate and coactivators interact with nuclear receptors in an agonist-dependent manner through conserved LXXLL motif (where X is any amino acid) (Heery et al., 1997; Torchia et al., 1997). This coactivator domain is oriented by a “charge clamp” formed by residues within helix 3 and the AF-2 of helix 12 of the LBD. It can then bind to a hydrophobic cleft in the surface of the receptor formed by helices 3, 4, and 5 and the AF-2 helix (Nolte et al., 1998b). Several coactivators, including CBP/p300 and steroid receptor coactivator (SRC)-1 (Zhu et al., 1996), possess histone acetylase activity that can remodel chromatin structure. A second group of co-activators represented by members of the DRIP/TRAP complex such as PPAR binding protein (PBP)/TRAP220 (Zhu et al.,

1997), form a bridge between the nuclear receptor and the transcription initiation machinery. The precise role of a third group, including PPAR $\gamma$  co-activator-1 (PGC-1) (Puigserver et al., 1998), receptor interacting protein 140 (RIP140) (Miyata et al., 1998), and androgen receptor associated protein 70 (ARA70) (Heinlein et al., 1999), is not well understood at the molecular level. At its most simple, a sequence of events can be envisioned in which coactivators with histone acetylase activity complex with PPRE-bound activated PPAR/RXR receptors, disrupt nucleosomes, and “open up” chromatin structure in the vicinity of the regulatory region of a gene. Complexes such as DRIP/TRAP are then recruited and provide a direct link to the basal transcription machinery. As a result, initiation of transcription is induced.

### ***Ligands***

PPARs are able to bind several structurally diverse natural and synthetic ligands due to their large ligand binding pocket (Table 1.4). The structures of these ligands are illustrated in Figure 1.22 (exogenous compounds) and Figure 1.23 (endogenous compounds). PPAR $\alpha$  is an important regulator of lipid metabolism a number of fatty acids and their derivatives bind and activate PPAR $\alpha$ - these include palmitic acid, oleic acid, linoleic acid, arachidonic acid (Gottlicher et al., 1992) and the lipoxygenase products LTB<sub>4</sub> and 8-S-HETE. The non-steroidal anti-inflammatory drugs (NSAIDs) indomethacin, fenoprofen, ibuprofen and flufenamic acid also act as ligands as well as the fibrate class of hypolipidaemic drugs which exert their therapeutic effect via this mechanism (Lehmann et al., 1997). Fatty acids are also thought to bind and activate PPAR $\delta$  although with a lower affinity than that to PPAR $\alpha$ . Synthetic agonists for PPAR $\delta$  include GW501516 and L-165041.

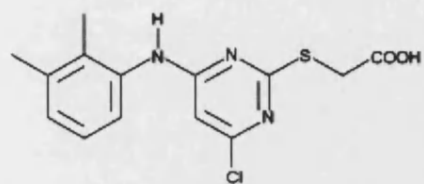
Some fatty acids and their derivatives can bind PPAR $\gamma$  with relatively low affinity; however it is believed that their relevant concentrations in the nuclei of target cells are likely to be too low for them to be *bona fide* ligands. Certain eicosanoids have been shown to bind and activate PPAR $\gamma$  with higher affinity (Yu et al., 1995; Reginato et al., 1998) such as 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), the prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) metabolite. However 15d-PGJ<sub>2</sub> has not been definitively proven to exist *in vivo*, nor are its effects specific to PPAR $\gamma$  (Li et al., 2001; Wilmer et al., 2001).

Several key observations made in the mid-1990s regarding thiazolidinedione (TZD) antidiabetic agents have allowed researchers to determine their primary molecular site of action. Such compounds have been developed over the preceding years on the basis of their insulin-sensitizing effects in pharmacological studies in animals. TZDs were found to induce adipocyte fatty acid-binding protein aP2 (Kletzien et al., 1992; Harris and Kletzien, 1994). Independently, Tontonoz et al. (1994), reported that PPAR $\gamma$  interacted with a regulatory element within the 5' flanking region of the aP2 gene that controlled its adipocyte-specific expression. These seminal observations were the precursor to additional experiments, which determined that TZDs such as rosiglitazone, pioglitazone, englitazone and ciglitazone were, in fact, PPAR $\gamma$  ligands and agonists (Lehmann et al., 1995; Berger et al., 1996; Willson et al., 1996). Such characterization of these anti-diabetic agents also demonstrated a definite correlation between the *in vivo* PPAR $\gamma$  binding and agonist activities of these compounds and their *in vivo* insulin-sensitizing actions. (Berger et al., 1996; Willson et al., 1996).

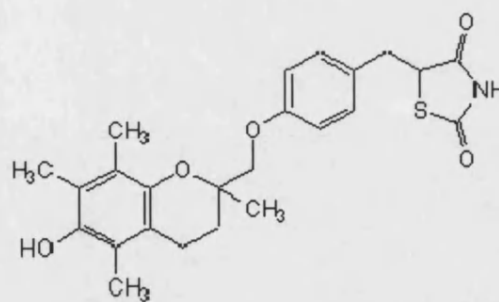
TZDs were developed primarily to improve the antidiabetic actions of the fibrate hypolipidemic agents. Several TZDs including troglitazone have insulin-sensitizing and antidiabetic activity in humans with type 2 diabetes or impaired glucose tolerance (Willson et al., 1996). Other synthetic ligands include NSAIDs and aryl-tyrosine derivatives.

	PPAR			
Ligands	$\alpha$	$\delta$	$\gamma$	Reference
<i>Exogenous compounds</i>				
WY-14,643	+++	+	++	(Desvergne and Wahli, 1999)
Troglitazone	-	-	+++	(Willson et al., 2000)
Rosiglitazone	-	-	+++	(Willson et al., 2000)
Ciglitazone	-	-	+++	(Yang and Frucht, 2001)
Pioglitazone	-	-	+++	(Willson et al., 2000)
Indomethacin	+	-	+++	(Lehmann et al., 1997)
Ibuprofen	+	-	+	(Lehmann et al., 1997)
<i>Endogenous activators</i>				
Palmitic acid	+++	++	-	(Xu et al., 1999a)
Linoleic acid	+++	++	+	(Xu et al., 1999a)
Arachidonic acid	+++	++	+++	(Xu et al., 1999a)
PGA <sub>1</sub>	+	++	+	(Yu et al., 1995)
PGD <sub>2</sub>	++	+	++	(Yu et al., 1995)
PGJ <sub>2</sub>	+	+/-	+++	(Yu et al., 1995)
15-Deoxy- $\Delta^{12,14}$ -PGJ <sub>2</sub>	+	+/-	+++	(Desvergne and Wahli, 1999)
8(S)-HETE	+++	-	-	(Desvergne and Wahli, 1999)
15-HETE	?	?	+++	(Nagy et al., 1998)
LTB <sub>4</sub>	+/-	?	?	(Desvergne and Wahli, 1999)
9-HODE	?	?	+++	(Nagy et al., 1998)
13-HODE	?	?	+++	(Nagy et al., 1998)

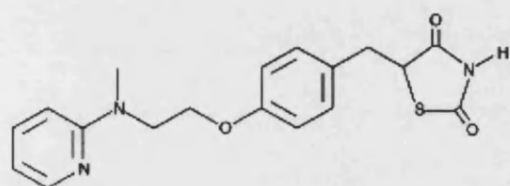
**Table 1.4. Activity of peroxisome proliferator-activated receptor ligands for receptor subtypes** (symbols are: +, activator; -, not an activator; +/-, possible activator; ?, not determined). Abbreviation: HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid.



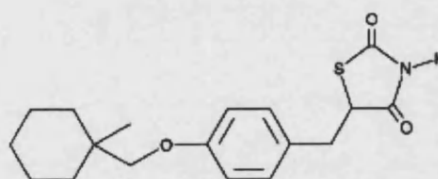
**WY-14643**



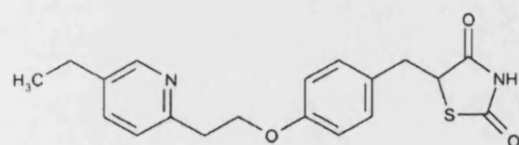
**Troglitazone**



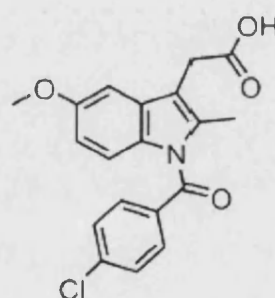
**Rosiglitazone**



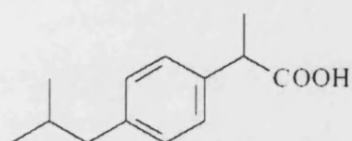
**Ciglitazone**



**Pioglitazone**



**Indomethacin**

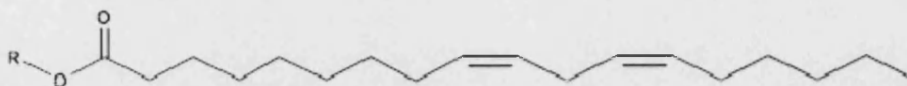


**Ibuprofen**

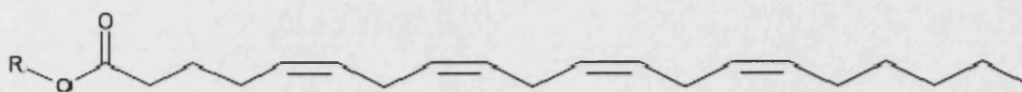
Figure 1.22. Structure of exogenous PPAR ligands



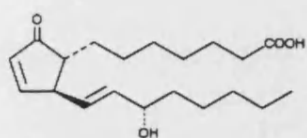
**Palmitic acid**



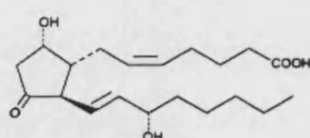
**Linoleic acid**



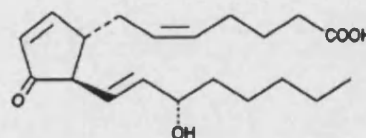
**Arachidonic acid**



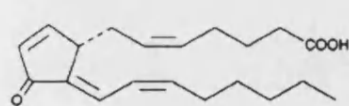
**PGA<sub>1</sub>**



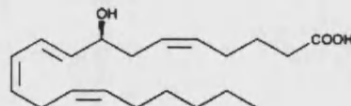
**PGD<sub>2</sub>**



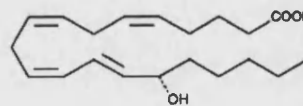
**PGJ<sub>2</sub>**



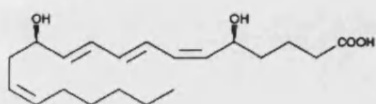
**15d-PGJ<sub>2</sub>**



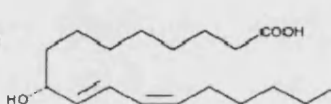
**8(S)-HETE**



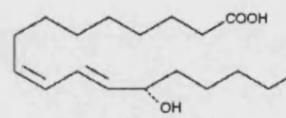
**15-HETE**



**LTB<sub>4</sub>**



**9-HODE**



**13-HODE**

**Figure 1.23. Structure of endogenous PPAR ligands**

### ***Expression***

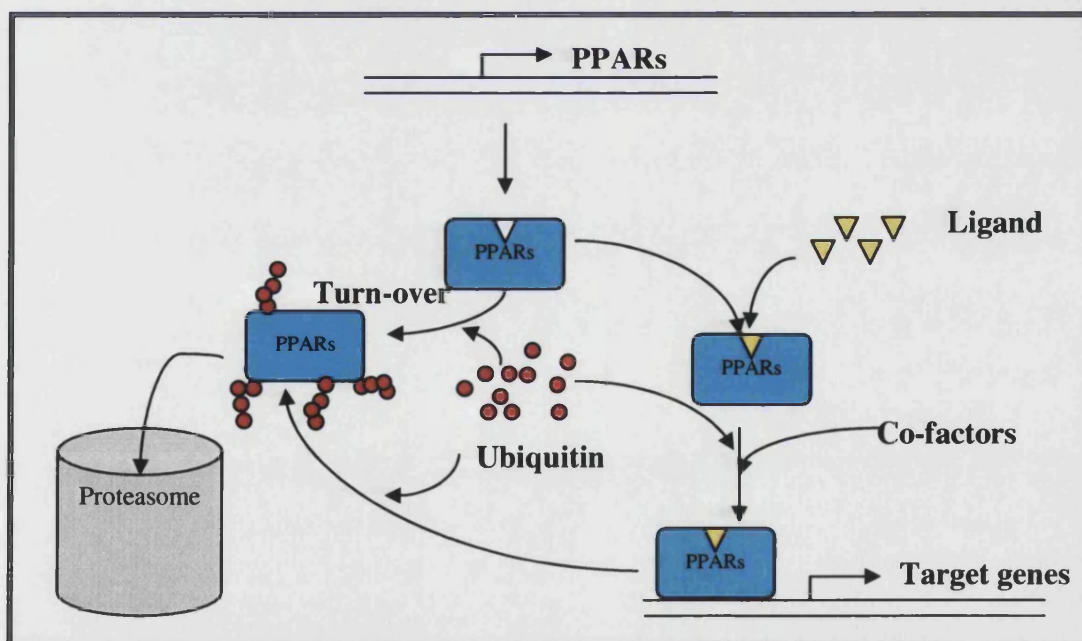
PPAR $\alpha$  is predominantly expressed in the liver and to a lesser extent in the heart and muscles. In comparison PPAR $\delta$  is abundantly expressed throughout the body but is at low levels in the liver (Evans et al., 2004). PPAR $\gamma$  is expressed in numerous metabolically active tissues including liver, kidney, heart, skeletal muscle, colon, small and large intestine, pancreas, spleen and brown fat. It is also present in monocytic, vascular endothelial and vascular smooth muscle cells (Berger and Moller, 2002). In relation to subtype distribution, PPAR $\gamma$ 2 is much less abundant in all tissues in comparison with PPAR $\gamma$ 1, the predominant form (Fajas et al., 2001). PPAR $\gamma$ 3 mRNA expression is restricted to macrophages and the large intestine (Fajas et al., 1998). Consistent with their expression profiles, the PPARs carry out unique functions in the regulation of energy metabolism.

### ***Ubiquitination***

Proteasomes act on proteins that have been specially marked for destruction by the covalent attachment of a small protein called ubiquitin. Ubiquitin exists in cells either free or covalently linked to proteins. Most ubiquitinated proteins have been tagged for degradation. Different ubiquitin-dependent proteolytic pathways employ structurally similar but distinct ubiquitin-conjugated enzymes that are associated with recognition subunits that direct them to proteins carrying a particular degradation signal. The conjugation enzyme adds ubiquitin to a lysine residue of a target protein and thereafter adds a series of additional ubiquitin moieties, forming a multiubiquitin chain that is thought to be recognized by a specific receptor protein in the proteasome. Recent data demonstrate that the ubiquitin-proteasome degradation



system affects the activity of several nuclear receptors. This degradation pathway is implicated in the regulation of many short-lived proteins involved in essential cellular functions, including cell cycle control, transcription regulation and signal transduction (Mimnaugh et al., 1999). Ligand-activation of PPAR $\gamma$  results in the degradation of this nuclear receptor via this pathway (Hauser et al., 2000). Interestingly, interaction with co-repressor proteins protect PPAR $\gamma$  from degradation whereas interaction with co-activators leads to enhanced degradation (Hauser et al., 2000). It has been postulated that ligands regulate PPAR degradation in a timely manner. In a first stage, the ligand may protect PPAR from degradation in order to increase the ligand effect, whereas in a second stage, agonist-induced AF-2 repositioning and cofactor recruitment may lead to PPAR ubiquitination and degradation as a mechanism to arrest transcriptional activation (Figure 1.24).



**Figure 1.24. Possible mechanisms of PPAR degradation by the ubiquitin–proteasome system.** PPAR proteins are degraded by the ubiquitin–proteasome pathway. This system controls the PPAR proteins level in cells and thus the intensity of the response to the ligand. However, first the ligand stabilizes the PPAR proteins by decreasing its ubiquitination and then the ligand induces the degradation of the PPAR proteins as the consequence of the cofactors recruitment and in order to stop the response (Blanquart et al., 2003).

Extracellular signals which activate intracellular phosphorylation pathways can also influence the degradation process, as shown for PPAR $\gamma$  (Floyd and Stephens, 2002). Treatment of cells with an inhibitor of MEK kinases inhibits the degradation of PPAR $\gamma$ . Thus PPAR degradation by the ubiquitin-proteasome pathway may be an important mechanism in the regulation of PPAR transcriptional activity by controlling cellular PPAR protein levels.

### **1.5.2. Role of PPAR $\alpha$ and PPAR $\delta$ in inflammation**

PPAR $\alpha$  plays a crucial role in controlling fatty acid oxidation. In a variety of mouse models, PPAR $\alpha$  agonists lower plasma triglycerides, reduce adiposity and improve hepatic and muscle steatosis, consequently improving insulin sensitivity (Chou et al., 2002; Kim et al., 2003). In terms of inflammatory responses once activated, PPAR $\alpha$  has been shown to inhibit the pro-inflammatory enzyme, COX-2 via a NF $\kappa$ B dependent pathway and has been hypothesised as having an anti-inflammatory action (Staels et al., 1998). In contrast the potent PPAR $\alpha$  ligand, WY-14,643, induced COX-2 protein in a breast epithelial cell line demonstrating the lack of consensus in this area (Meade et al., 1999).

Genetic studies and recently developed synthetic PPAR $\delta$  agonists have helped reveal the role of PPAR $\delta$  as a powerful regulator of fatty acid catabolism and energy homeostasis (Peters et al., 2000; Barak et al., 2002). Little is known of the physiological role of PPAR $\delta$  in terms of inflammation, although it has been shown to act as a receptor for the COX-2 product PGI<sub>2</sub> in a model of blastocyst

implantation ( Lim et al., 1999; Meade et al., 1999). It is also emerging as having a potential role in colorectal carcinogenesis (DuBois et al., 1998) where its mRNA is seen to co-localise with that for COX-2 although the full implications of this finding are yet to become clear. The role of PPAR $\gamma$  in inflammation has recently become of interest and is the focus of this thesis, thus the next section will introduce PPAR $\gamma$  and inflammation in more detail.

### **1.5.3. Role of PPAR $\gamma$ in inflammation**

#### ***PPAR $\gamma$ and anti-inflammatory effects***

##### ***In vitro effects***

An involvement of PPAR $\gamma$  in inflammatory processes was first suggested by the antagonism between the activities of the proinflammatory cytokine TNF $\alpha$  on the one hand and PPAR $\gamma$  on the other hand (Peraldi et al., 1997). Consistent with this were observations that PPAR $\gamma$  agonists seem to inhibit macrophage activation (Ricote et al., 1998a) and limit the production of cytokines (Jiang et al., 1998) and chemokines (Marx et al., 2000), suggesting an inhibitory role in both inflammation and migration of immune cells. PPAR $\gamma$  expression is dramatically up-regulated in macrophages and T cells during inflammatory responses, and can be induced by IL-4 and other immunoregulatory molecules (Ricote et al., 1998b; Huang et al., 1999; Daynes and Jones, 2002). In addition, airway inflammation and alterations in cellular turnover are histopathologic features of asthma and PPAR $\gamma$  expression is augmented in the

bronchial submucosa, airway epithelium and smooth muscle of steroid-untreated asthmatics, as compared with control subjects (Benayoun et al., 2001).

PPAR $\gamma$  ligands have shown to inhibit production of many pro-inflammatory mediators such as TNF $\alpha$ , IL-6, IL-1 $\beta$  and monocyte chemoattractant protein (MCP)-1 expression in monocytes (Jiang et al., 1998; Zhang et al., 2001); iNOS, gelatinase B and scavenger receptor-A expression in macrophages (Colville-Nash et al., 1998; Ricote et al., 1998b); IFN-inducible protein 10, monokine induced by interferon gamma, interferon gamma inducible T-cell alpha chemoattractant, endothelin-1, IL-8 and MCP-1 expression in endothelial cells (Delerive et al., 1999b; Lee et al., 2000; Marx et al., 2000); IL-2 in T lymphocytes (Yang et al., 2000); IL-8 in colonic epithelial cells (Su et al., 1999) and iNOS and IL-8 in airway epithelial cells (Wang et al., 2001).

Expression of PPAR $\gamma$  has also been shown to be robustly upregulated upon the differentiation of monocytes into macrophages (Chinetti et al., 1998). Down regulation of immunity can be induced by apoptosis, and ligand activation of PPAR $\gamma$  has been shown to result in apoptosis of nonactivated and activated macrophages (Chinetti et al., 1998).

Furthermore, the chemokine RANTES is a potent chemoattractant for eosinophils. RANTES is produced by lung epithelial cells during eosinophil-rich inflammatory diseases such as asthma. In A549 cells, IL-1 $\beta$  and TNF $\alpha$  induce mRNA expression, endogenous RANTES protein secretion, and promoter activity, which have been

shown to be inhibited by TZDs. This raises the possibility that TZDs might be of therapeutic value in diseases such as asthma (Momoi et al., 1999).

### *In vivo effects*

Several studies have investigated the role of PPAR $\gamma$  ligands in modifying animal models of autoimmune diseases. Su et al. (1999), showed that in a mouse model of inflammatory bowel disease, TZDs markedly reduced colonic inflammation. Kawahito et al. (2000), demonstrated that intraperitoneal administration of PPAR $\gamma$  ligands ameliorated adjuvant-induced arthritis with suppression of pannus formation and mononuclear cell infiltration in rats. 15d-PGJ<sub>2</sub> exerts potent anti-inflammatory effects in the carrageenan-induced pleurisy and collagen-induced arthritis models of acute and chronic inflammation, respectively (Cuzzocrea et al., 2003). 15d-PGJ<sub>2</sub> and PPAR $\gamma$  ligands have also been shown to inhibit inflammation in models of ischemia-reperfusion injury (Nakajima et al., 2001). Although in many of these studies, when more selective ligands (e.g. TZDs) were used, high concentrations (far exceeding those required to bind the receptor) were necessary to achieve the results described (Chawla et al., 2001). In addition, PPAR $\gamma$  has been shown to inhibit inflammation in a mouse model of atherosclerosis (Li et al., 2000a). Woerly et al. (2003), demonstrated that PPAR $\gamma$  was expressed by eosinophils and that PPAR $\gamma$  agonists were able to inhibit eosinophil chemotaxis and antibody-dependent cellular cytotoxicity reactions *in vitro*. Furthermore, in a murine model of asthma, treatment with a PPAR $\gamma$  agonist also inhibited the development of allergic inflammation, including pulmonary eosinophilia and airway hyperresponsiveness. However, target

cells for PPAR $\gamma$  agonists within the airways and the mechanisms by which they inhibit allergic inflammation were poorly characterised (Woerly et al., 2003). Moreover, Mueller et al. (2003), not only showed that PPAR $\gamma$  ligands reduce the immunological symptoms of allergic asthma, such as reduced lung inflammation and mucus production, in a murine model of this disease, but that T cells from these ciglitazone treated mice also produce less IFN $\gamma$ , IL-4 and IL-2 upon rechallenge *in vitro* with the model allergen.

### ***PPAR $\gamma$ and pro-inflammatory effects***

Despite extensive evidence suggesting the anti-inflammatory effects of PPAR $\gamma$  ligands, they have also been found to stimulate the expression of the proinflammatory receptors CD14 and CD11b/CD18 and to increase expression of class B scavenger receptors (CD36 and SR-B1) (Nagy et al., 1998; Tontonoz et al., 1998; Chinetti et al., 2000). 15d-PGJ<sub>2</sub> can induce expression of the proinflammatory mediators type II secreted phospholipase A<sub>2</sub> and COX-2 in smooth muscle and epithelial cells, respectively (Couturier et al., 1999; Meade et al., 1999). The regulation of COX-2 by PPAR $\gamma$  ligands will be outlined in more detail further on in this section. 15d-PGJ<sub>2</sub> can also stimulate the production of proinflammatory mediators, such as IL-8 and MAPK, in some systems (Wilmer et al., 2001; Zhang et al., 2001; Fu et al., 2002). Thus, it is clearly evident that PPAR $\gamma$  ligands, but in particular, 15d-PGJ<sub>2</sub>, possess both anti-inflammatory and pro-inflammatory functions *in vitro* (which are summarised in Table 1.5). In addition, plasma TNF $\alpha$  levels following challenge with LPS (Thieringer et al., 2000) or the development of arthritis (Wiesenberg et al., 1998) were not affected by PPAR $\gamma$  ligand treatment.

Therefore further investigation is required in order to identify the complex role of PPAR $\gamma$  ligands in the regulation of inflammation.

	ANTI-INFLAMMATORY	PRO-INFLAMMATORY
<u>Monocytes</u>	↓ <b>TNF<math>\alpha</math>, IL-6, IL-1<math>\beta</math> and MCP-1</b> (Jiang et al., 1998; Zhang et al., 2001)	↑ <b>IL-8</b> (Wilmer et al., 2001; Zhang et al., 2001; Fu et al., 2002)
<u>Macrophages</u>	↓ <b>iNOS (inducible nitric oxide), gelatinase B and scavenger receptor-A</b> (Colville-Nash et al., 1998; Ricote et al., 1998a)	↑ <b>expression of the proinflammatory receptors CD14 and CD11b/CD18 and class B scavenger receptors</b> (Nagy et al., 1998; Tontonoz et al., 1998; Chinetti et al., 2000) <b>IL-8 and MAPK</b> (Wilmer et al., 2001; Zhang et al., 2001; Fu et al., 2002).
<u>Endothelium</u>	↓ <b>IFN-inducible protein 10, monokine induced by interferon gamma, interferon gamma inducible T-cell alpha chemoattractant, endothelin-1, IL-8 and MCP-1 expression</b> (Delerive et al., 1999b; Lee et al., 2000; Marx et al., 2000)	
<u>T-lymphocytes</u>	↓ <b>IL-2</b> (Yang et al., 2000)	
<u>Colonic epithelial cells</u>	↓ <b>IL-8</b> (Su et al., 1999)	
<u>Airway epithelial cells</u>	↓ <b>iNOS, IL-8 and RANTES</b> (Wang et al., 2001).	↑ <b>COX-2</b> (Meade et al., 1999)

Table1.5. Summary of the anti- and pro-inflammatory effects of PPAR $\gamma$  *in vitro*

#### 1.5.4. Molecular mechanisms of the anti-inflammatory effects of PPAR $\gamma$ ligands

##### *PPAR $\gamma$ - dependent mechanisms*

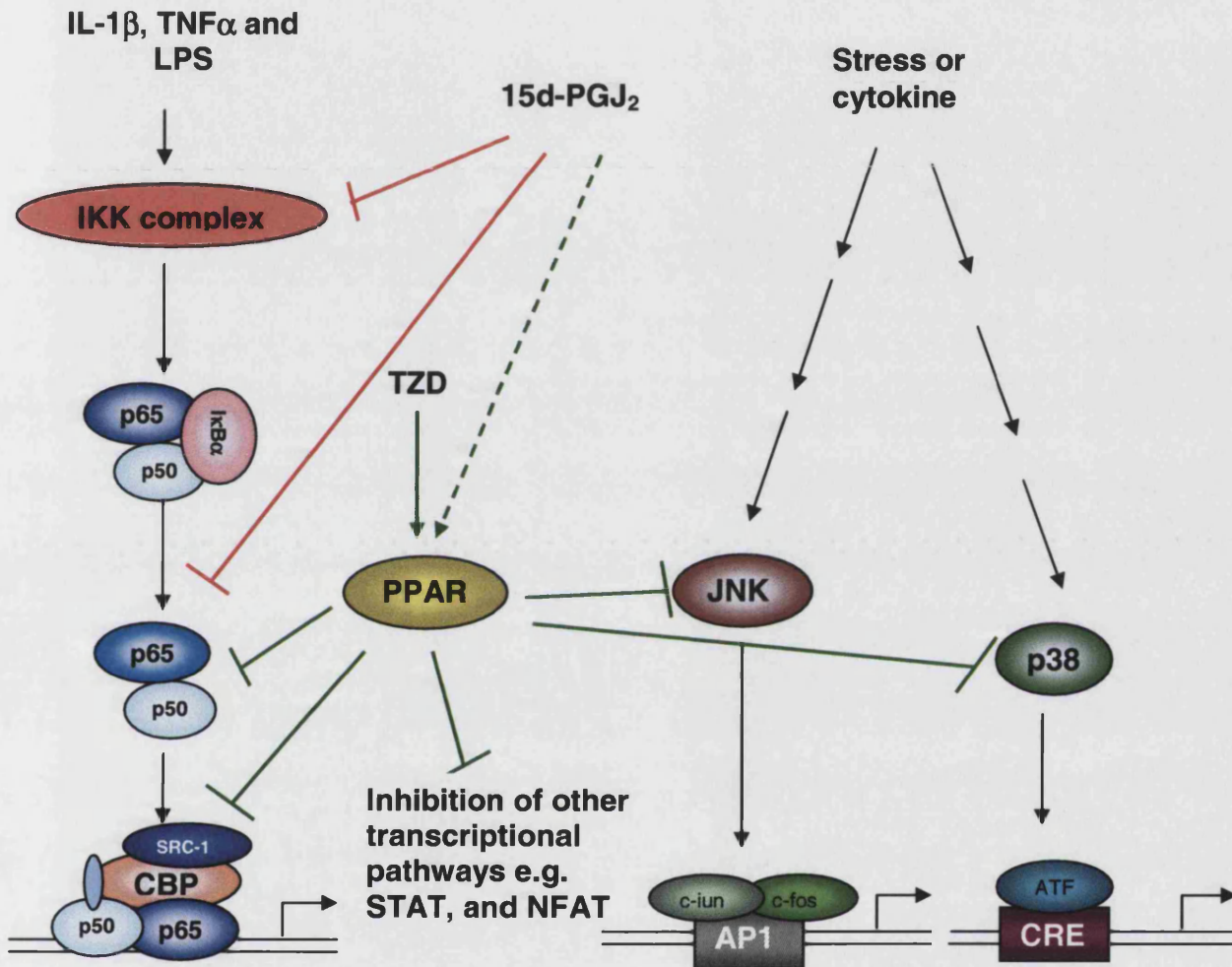
The molecular mechanisms by which PPAR $\gamma$  regulates inflammatory response genes are not yet fully understood. In addition to the classical mechanism of gene

regulation (described above), PPAR $\gamma$  can inhibit NF $\kappa$ B-driven transcription by physically interacting with both p65 (Chung et al., 2000) and p50 (Cernuda-Morollon et al., 2001). Using endothelin-1 promoter as a model, it has been demonstrated that PPAR $\gamma$  inhibits AP-1 transcriptional activity by reducing AP-1 DNA binding and thus the production of endothelin-1 (Delerive et al., 1999b). This inhibition is likely due to a direct interaction between PPAR $\gamma$  and c-jun as previously reported for PPAR $\alpha$  (Delerive et al., 1999a). Law et al. (1996), reported that TZDs inhibit c-fos transcription in vascular smooth muscle cells. In a model of cardiac ischemia reperfusion, rosiglitazone significantly reduced c-jun-NH<sub>2</sub>-terminal kinase (JNK) activation *in vivo* (Delerive et al., 2001). Previous reports suggested that nuclear receptors may regulate AP-1 activation by modulating JNK function (Caelles et al., 1997; Srivastava et al., 1999). However little is known about the molecular mechanisms of kinase inhibition by nuclear receptors.

A model for PPAR $\gamma$ -mediated inhibition of iNOS transcription was proposed by Li et al. (2000b). In this model, it was suggested that PPAR $\gamma$  inhibits STAT1, AP-1 and NF $\kappa$ B transcriptional activities by targeting coactivators such as CBP through direct interaction with its N-terminal domain and via SRC-1-like bridge factors (Li et al., 2000b). Such a model of competition for limiting amounts of co-activators to inhibit transcriptional activation has already been proposed for other nuclear receptors (Kamei et al., 1996; Sheppard et al., 1998). However, this hypothesis of transrepression is not supported by recent reports (De Bosscher et al., 2000; McKay and Cidlowski, 2000). Finally, Yang et al. (2000), showed that PPAR $\gamma$  activation results in a reduction of IL-2 secretion in T lymphocytes, which is due to a ligand



dependent interaction between the transcription factor nuclear factor of activated T-cells (NFAT) and PPAR $\gamma$ . Figure 1.25 outlines the molecular mechanism of anti-inflammatory effects of PPARs.



**Figure 1.25. Hypothetical model of PPAR $\gamma$ -dependent and independent inhibition of the inflammatory response genes (modified from (Delerive et al., 2001)).** 15d-PGJ<sub>2</sub> may inhibit transcription of inflammatory response genes independent of PPAR $\gamma$  (shown by red lines) by inhibiting the IKK complex and thereby preventing activation of NF $\kappa$ B and subsequent transcription, or by directly inhibiting NF $\kappa$ B (p65/p50). 15d-PGJ<sub>2</sub> and TZDs may also prevent transcription in a PPAR $\gamma$  dependent mechanism (shown by green lines), by directly inhibiting NF $\kappa$ B or binding to and competing for cofactors required by NF $\kappa$ B mediated transcription. TZD and 15d-PGJ<sub>2</sub> may also cause inhibition of other transcriptional pathways, such as STAT, NFAT, JNK or p38.

### ***PPAR $\gamma$ independent mechanisms***

Many studies have indicated that 15d-PGJ<sub>2</sub> may not be specific for PPAR $\gamma$  (Petrova et al., 1999; Hortelano et al., 2000; Tsubouchi et al., 2001). In contrast to the results

described above, PPAR $\gamma$  ligands, with the exception of 15d-PGJ<sub>2</sub>, do not inhibit LPS-induced cytokine production in cultured macrophages and diabetic *db/db* mice treated *in vivo* (Thieringer et al., 2000). Chawla et al. (2001), recently utilized murine PPAR $\gamma$  null macrophages to demonstrate that previously reported inhibitory actions of PPAR $\gamma$  agonists on macrophage cytokine production occur via a receptor-independent mechanism. 15d-PGJ<sub>2</sub> has been shown to suppress the production of iNOS in activated microglia and astrocytes by a mechanism independent of PPAR $\gamma$  activation (Petrova et al., 1999). This was shown by the fact that 15d-PGJ<sub>2</sub> could not stimulate the activity of a PPAR-dependent promoter in the absence of cotransfected PPAR $\gamma$  in these cells. These pharmacological discrepancies suggested that 15d-PGJ<sub>2</sub> may act through PPAR $\gamma$ -independent pathways. Reports indeed demonstrate that, in the absence of PPAR $\gamma$  expression, 15d-PGJ<sub>2</sub> also negatively regulates the inflammatory response (Petrova et al., 1999).

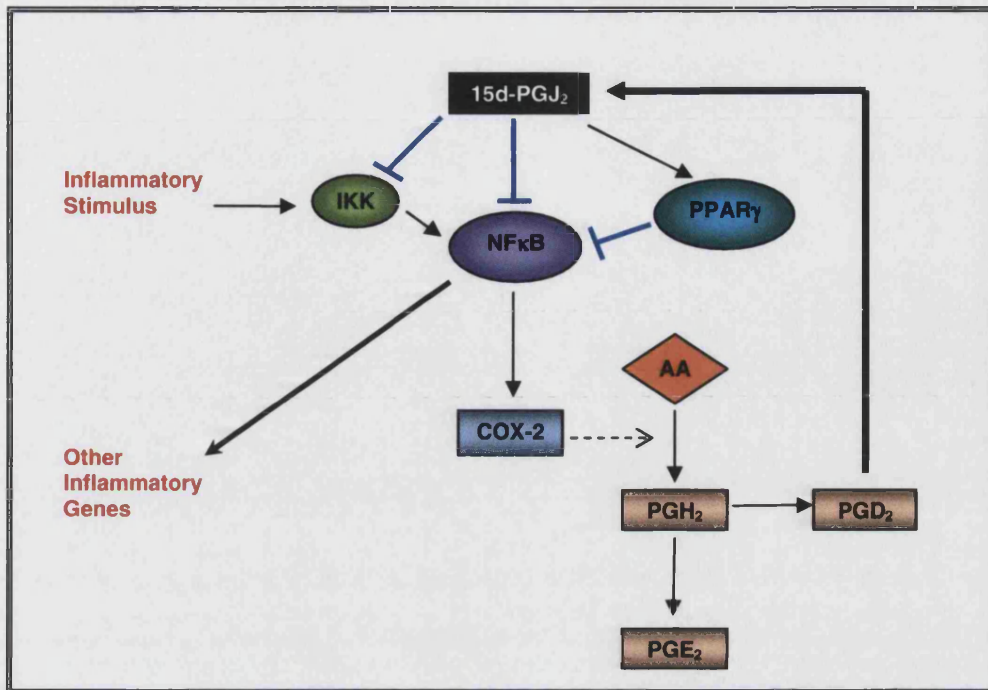
There is abundant evidence implicating NF $\kappa$ B as a major target for receptor-independent gene repression by 15d-PGJ<sub>2</sub> (Castrillo et al., 2000; Straus et al., 2000; Boyault et al., 2001). It has been demonstrated that 15d-PGJ<sub>2</sub> can directly inhibit and modify the IKK2 subunit of IKK. This, in turn, prevents the phosphorylation of the inhibitory I $\kappa$ B proteins thus preventing the activation of NF $\kappa$ B (Castrillo et al., 2000; Rossi et al., 2000). 15d-PGJ<sub>2</sub> has also been shown to directly inhibit binding of NF $\kappa$ B to target DNA sequences (Straus et al., 2000) (Figure 1.25). Both mechanisms for inhibition of NF $\kappa$ B activity involve the alkylation of specific cysteine residues in target proteins. Furthermore, Chinetti et al. (1998), demonstrated that rosiglitazone induced apoptosis of cultured macrophages by altering NF $\kappa$ B signalling at

concentration that paralleled its known affinity for PPAR $\gamma$ . This ligand has also been shown to block inflammatory cytokine synthesis in colonic cell lines by inhibiting activation of the NF $\kappa$ B pathway (Su et al., 1999). This latter observation offers a possible mechanistic explanation for the observed anti-inflammatory actions of TZDs in rodent models of colitis (Su et al., 1999)

#### 1.5.5. PPAR $\gamma$ and the COX-2 pathway

##### *Negative feedback regulation of prostaglandin synthesis via PPAR $\gamma$ and NF $\kappa$ B*

15d-PGJ<sub>2</sub> may function as a physiological negative feedback regulator of prostaglandin synthesis and inflammatory response. As previously outlined, 15d-PGJ<sub>2</sub> is present in the inflammatory fluids during the resolution phase of inflammation (Gilroy et al., 1999). PPAR $\gamma$  is expressed at high levels in activated macrophages, making these cells likely targets for receptor-dependent actions of 15d-PGJ<sub>2</sub> (Ricote et al., 1998b). Finally, 15d-PGJ<sub>2</sub> represses the synthesis of COX-2 and inhibits the production of PGE<sub>2</sub> by macrophages, consistent with the hypothesis that it can act as a negative feedback regulator of prostaglandin synthesis (Straus et al., 2000). Thus 15d-PGJ<sub>2</sub> participates in a physiological negative feedback loop by repressing a number of inflammatory response genes (e.g. iNOS and TNF $\alpha$ ) as well as the synthesis of the inflammatory mediator PGE<sub>2</sub> via inhibition of NF $\kappa$ B (Figure 1.26).



**Figure 1.26. Model for mechanisms by which 15d-PGJ<sub>2</sub> exerts negative feedback regulation of prostaglandin biosynthesis and inflammatory gene expression.** During an inflammatory response, COX-2 produces prostaglandins from arachidonic acid. Many of these prostaglandins are involved in inflammation. PGD<sub>2</sub> can be dehydrated to produce the metabolite 15d-PGJ<sub>2</sub> which can then inhibit further COX-2 expression by various mechanisms (AA-arachidonic acid).

### COX-2 and PPAR

The PPAR $\gamma$  ligand and metabolite of COX-2 product, 15d-PGJ<sub>2</sub>, has been shown to suppress LPS-induced expression of COX-2 in macrophage-like differentiated U937 cells (Inoue et al., 2000); COX-2 induction in astrocytes (Janabi, 2002); and PMA induced COX-2 expression and PGE<sub>2</sub> synthesis in human epithelial cells (Subbaramaiah et al., 2001). In contrast PPAR $\gamma$  ligands have also been shown to increase TNF- $\alpha$  induced COX-2 expression in human colonic epithelial cells (Ikawa et al., 2001).

PPAR $\gamma$  activated by its ligands can block both AP-1 and NF $\kappa$ B mediated COX-2 gene expression (Inoue et al., 2000). The mechanism of suppression by PPAR $\gamma$  is a

consequence of inhibition of c-jun expression and competition for limiting amounts of the general coactivator CREB-binding protein (CBP)/p300. Binding of a PPAR $\gamma$  ligand enhances the interaction between CBP/p300 and PPAR $\gamma$  (Mizukami and Taniguchi, 1997; Gelman et al., 1999). This results in less CBP/p300 being available for AP-1 mediated induction of the COX-2 gene.

NSAIDs are widely used in the treatment of inflammation, and their efficacy is considered to be a result of their inhibitory effects on COX-2. However, NSAIDs have recently been identified as ligands of PPARs. Paik et al. (2000), reported that the NSAID, flufenamic acid, showed two opposing effects on COX-2 expression; it induces COX-2 expression in colonic epithelial cells and macrophages; conversely, it inhibits TNF $\alpha$  or LPS-induced COX-2 expression, and similar effects were also seen with 15d-PGJ<sub>2</sub>. These results indicated that NSAIDs inhibit mitogen-induced COX-2 expression while they induce COX-2 expression (Paik et al., 2000).

Due to the disparate effects seen on COX-2 expression in different systems, it is difficult to fully dissect out the regulatory mechanisms of PPAR $\gamma$  on COX-2 expression.

#### **1.5.6. PPAR $\gamma$ and signalling pathways**

##### ***PI3K and PPAR***

Insulin is a strong activator of PI3K/PKB dependent pathway, and TZD drugs are clinically used to treat type 2 diabetes since they can increase insulin sensitivity. Rieusset et al. (2001a;2001b), demonstrated that the gene of p85 $\alpha$ PI3K is a likely

target of PPAR $\gamma$  in normal human adipocytes and PPAR $\alpha$  in human muscle cells. However, p85 k/o are more susceptible to hypoglycaemic have increased insulin sensitivity (Fruman et al., 2000; Ueki et al., 2002). Furthermore, the PI3K/PKB pathway has been shown to be involved in downstream signalling of PPAR $\gamma$  ligands in various pathways such as the cell migration of human umbilical vein endothelial cells (Goetze et al., 2002). In addition, PKB has been linked to the regulation of NF $\kappa$ B and/or Rel family transcription factors that are essential for the induction of cytokine gene expression (Kane et al., 1999). Thus TZD and other PPAR $\gamma$  ligands may induce PI3K isoforms in human lung epithelial cells and may affect PPAR $\gamma$  mediated transcription of pro-inflammatory cytokines. 15d-PGJ<sub>2</sub> has been shown to suppress inflammatory response by inhibiting NF $\kappa$ B signaling at multiple steps as well as by inhibiting the PI3K/Akt pathway independent of PPAR $\gamma$  in primary astrocytes (Giri et al., 2004). 15d-PGJ<sub>2</sub> induction of HO-1 gene expression in human lymphocytes was also shown to involve the PI3K signaling pathway (Alvarez-Maqueda et al., 2004).

### ***MAPK and PPAR***

15d-PGJ<sub>2</sub> and members of the TZD class of drugs have recently been shown to activate the MEK/ERK pathway in vascular smooth muscle cells (Takeda et al., 2001), in astrocytes and preadipocytes (Lennon et al., 2002) and human mesangial cells (Wilmer et al., 2001). Lennon et al. (2002), also showed the activation of the p38 and JNK MAPK pathways following stimulation with ciglitazone in astrocytes and preadipocytes. All these cells express PPAR $\gamma$ , however, the effects described above on ERK induction may possibly be mediated independently of PPAR $\gamma$  since

the time courses for the activation of ERK are too rapid to encompass the classical transcriptional mechanism (Takeda et al., 2001).

Recently, it has been demonstrated that the transcriptional activities of PPARs are regulated by post-translational mechanisms including phosphorylation and ubiquitination. In 1996, Zhang et al., showed that insulin treatment increases the ligand-independent transcriptional activity of PPAR $\gamma$  and synergizes with a PPAR $\gamma$  ligand to enhance its transcriptional activity. Moreover, the authors demonstrated that PPAR $\gamma$  is phosphorylated by the MAPK pathway *in vivo* (Zhang et al., 1996). In contrast, it has been shown that phosphorylation of mouse PPAR $\gamma$  by activators of MAPK induces an inhibition of its transcriptional activity (Hu et al., 1996). This result was confirmed in 1997 in a study by Adams et al., in which it was shown that serine 84 of human PPAR $\gamma$ 1 is phosphorylated by the MAPK ERK2 and JNK (Adams et al., 1997). Furthermore, mutation of this serine increases the AF-1 transcriptional activity of PPAR $\gamma$ . Camp et al. (1999), demonstrated that mouse PPAR $\gamma$ 1 is also regulated by MAPK phosphorylation at a site located on serine 82. Moreover these authors demonstrated that JNK, a MAPK, phosphorylates PPAR $\gamma$ 2 and decreases its ligand-dependent transcriptional activity. Han et al.(2002), demonstrated that treatment of macrophage with TGF $\beta$ 1 and 2 can inhibit PPAR $\gamma$  ligand-induced expression of CD36 via activation of the MAPK pathway.

## **1.6. Aim of the study**

For many years COX-2 has been a therapeutic target for the development of anti-inflammatory drugs for various diseases. COX-2 is generally regarded as a pro-inflammatory enzyme and thus regulating it can reduce inflammation. Furthermore PPAR $\gamma$  ligands are thought to be anti-inflammatory and have been shown to regulate COX-2 in other systems, thus the aim of this work was to investigate the mechanisms involved in the regulation of COX-2 by PPAR $\gamma$  ligands in lung epithelial cells and to assess whether PPAR $\gamma$  may be beneficial in the treatment of inflammatory lung diseases such as asthma and COPD. It was hypothesised that the PI3K pathway plays an important role in the regulation of COX-2.

The first results chapter investigates the signalling pathways activated by cytokines (TNF $\alpha$  and IL-1 $\beta$ ) and the involvement of these pathways in COX-2 regulation in the human lung epithelial cell line, A549. These cytokines were utilised since they have been shown to be important in inducing inflammatory responses as previously outlined. In order to investigate the role of PI3K pathway, pharmacological methods were employed. Previous work has shown that PI3K inhibitors, LY294002 and wortmannin, are poor inhibitors of recombinant PI3K-C2 $\alpha$  (Domin et al., 1997). Thus the sensitivity of PI3K isoforms to LY294002 and wortmannin was first characterised. It was important to dissect out the mechanisms and pathways involved in the regulation of COX-2 expression by cytokines since the second aim was to investigate the effects of PPAR $\gamma$  ligands on cytokine induced events.



Despite extensive studies utilizing PPAR $\gamma$  ligands, the mechanism of action of these compounds is not only ambiguous but varies from system to system. The next aim was to clarify the signalling pathways activated by PPAR $\gamma$  ligand in the A549 cell line and to investigate the involvement of these pathways in the regulation of COX-2, a known downstream target for activated PPAR $\gamma$  in other cell types. Understanding the mechanisms of action of these ligands in this cell line may reveal important therapeutic targets for inflammatory lung diseases.

## **2.**

# **METHODS & MATERIALS**

## **2. METHODS & MATERIALS**

### **2.1 Cell Culture Conditions**

#### **2.1.1 A549 cells**

The human lung epithelial cell line A549 was obtained from the European Collection of Animal Cell Cultures (ECACC). A549 cells are human lung epithelial adenocarcinoma cells isolated from a 58 year old Caucasian male (ECACC). A549s were routinely cultured in 175cm<sup>2</sup> tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (10 u/ml), streptomycin (10µg/ml), 10mM L-glutamine, 20mM HEPES buffer and 10% v/v FBS (referred to as complete medium). Cultures were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub>. The medium was changed every 2-3 days.

To subculture confluent monolayers, the medium was removed and the cells were washed twice with PBS (w/o Ca<sup>2+</sup> and Mg<sup>2+</sup>). Cells were then washed with a 3ml Trypsin-EDTA mixture of 0.05% (w/v) Trypsin and 0.02% (w/v) EDTA. The excess solution was removed and the cells were incubated for approximately 5 mins at 37°C until the cells had detached from the flask. The action of trypsin/EDTA was inhibited by the addition of complete DMEM medium and the cell suspension was centrifuged at 1500g for 5 min. The cell pellet was resuspended in complete medium and cell counting and viability were checked in a Neubauer haematocytometer after mixing 50 µl of cell suspension with 50 µl of Trypan Blue (Sigma). Dead cells stained blue, due to the uptake of Trypan Blue. Cell viability was always greater than 95%. Cells were counted and then seeded into 24-well

plates for luciferase assays; 6 well plates or 35mm petri dishes for western and ELISA protocols; 6cm (diameter) petri dishes for *in vitro* lipid kinase assays and nuclear extractions; and into 175 cm<sup>2</sup> for further culture. Flasks and plates reached confluency after approximately 3-4 days.

### 2.1.2 Jurkat cells

The human leukaemic T-cell line Jurkat was obtained from Imperial Cancer Research Fund. Jurkat were routinely cultured in 175cm<sup>2</sup> tissue culture flasks in RPMI 1640 medium supplemented with penicillin (10 u/ml), streptomycin (10µg/ml) and 10% v/v foetal calf serum (FBS) (referred to as complete medium). Cultures were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub>. Fresh medium was added every 2-3 days and confluent flasks were split into 3 175cm<sup>2</sup> flasks with fresh medium. Prior to cell stimulations, cells were washed three times in RPMI, counted, resuspended at a density of  $2 \times 10^7$  cells per 500µl for *in vitro* lipid kinase assays in FBS-free RPMI.

### 2.1.3. HT-29 cells

The human colonic epithelial cell line HT-29 was obtained from the European Collection of Animal Cell Cultures (ECACC). HT-29 cells are human colon adenocarcinoma grade II cells isolated from a primary tumour in a 44 year old Caucasian female. HT-29 colonic epithelial cells were routinely cultured in 80cm<sup>2</sup> tissue culture flasks in McCoy's medium supplemented with penicillin (10 u/ml), streptomycin (10 µg/ml), fungizone (0.5 µg/ml), and 10% (v/v) FBS (referred to as complete medium). Cultures were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub>.

The medium was changed every 2-3 days and cells were subcultured in a similar manner to A549 cells (described above).

#### 2.1.4. Freezing and Thawing of cells

For storage, cells were resuspended at  $4 \times 10^6$  cells/ml of freeze medium. The freeze medium contained 10% of dimethylsulphoxide (DMSO) (Sigma) and 90% FBS. The cell suspension was transferred to cryotubes (Nunc) at 1 ml/tube, gradually cooled in vapour phase of liquid nitrogen overnight and then stored in liquid nitrogen tanks. For resuscitation of cells from liquid nitrogen, cells were rapidly defrosted at 37°C in a water bath, washed in with their appropriate medium, resuspended in complete medium and A549 and Jurkat cells from 1 cryotube were seeded into 75cm<sup>2</sup> tissue culture flasks in complete DMEM and RPMI medium respectively. Tissue culture was then continued as above.

## 2.2. Experimental Protocol

A549 epithelial cells were cultured in the container appropriate to the planned experiment, as described above (Table 2.2.1).

Assay	Culture Vessel	Cell Number A549
Luciferase assays	24 well plates	$3.5 \times 10^6$ cells
Western analysis	6 well plates (6hr plus time points) 35mm dishes (up to 2 hr time points) 60mm dishes for nuclear extracts	$10^7$ cells
ELISAs	6 well plates	$10^7$ cells
In vitro lipid kinase assays	60mm dishes	

Table 2.1 Outline of the culture vessels used for various experimental protocols.

Prior to the experiment the confluent monolayers of A549 cells were washed and cultured in FBS-free medium overnight. Growth arrested cultures were then treated with fresh FBS-free medium and stimulated with the appropriate concentrations of either drugs, or cytokines, or vehicle controls for the times described in the results section. Supernatants for ELISAs were collected, centrifuged to remove cellular debris and stored at -80°C until assayed. Cellular and nuclear proteins were extracted as described below for Western analysis and for NFκB p65 activation assays. Cell counting and viability were routinely checked at the beginning and the end of the experiment, by phase microscopy and by trypan blue exclusion, using representative wells. Cell viability was always greater than 95%. Recipes for buffers and solutions are listed in the appendix.

## **2.3. Immunoblotting**

### **2.3.1. Overview**

Western analysis is a technique of assaying the relative amounts of specific proteins within cells. Once the cells have been incubated in the appropriate conditions the cells are lysed and the whole cell proteins are extracted and quantified. The whole cell extracts can then be run in a current through a polyacrylamide gel to separate the proteins according to their molecular weight. The proteins can then be transferred to a membrane which can be incubated with a primary antibody specific for the protein to be assessed. A second incubation with a labelled secondary antibody against the primary antibody is then carried out. The membrane is then washed and the relative amount of the labelled secondary antibody is then assessed, usually by using a horse

radish peroxidase tag on the secondary antibody to catalyse a reaction which can then be visualised.

From the point of view of the primary antibodies that are used, they can be specific for certain conformations of the protein to be assayed. In particular they can be directed to a phosphorylated form of the protein where phosphorylation indicates a change in function such as activation. Such an antibody can then be used to assess relative changes in the amount of this phosphorylated form of the protein, and by inference is a surrogate marker of any associated change in function. To ensure that the total amount of the specific protein is unchanged the results from these phosphorylated antibodies are compared to results using an antibody against all forms of the specified protein, referred to as a pan antibody.

### **2.3.2. Collecting samples**

Attached monolayers of A549 cells were stimulated and incubated at 37°C as described. Stimulations were terminated at the appropriate times with aspiration of the supernatant and then the addition of 200 µl ice cold lysis buffer (1% (v/v) Nonidet P-40, 150mM NaCl, 50mM Tris pH 7.5, 5mM EDTA, 10mM sodium fluoride, 1mM phenylmethylsulfonyl fluoride, 10µg ml<sup>-1</sup> leupeptin, 10µg ml<sup>-1</sup> aprotinin, 1µg ml<sup>-1</sup> soybean trypsin inhibitor, 1µg ml<sup>-1</sup> pepstatin A, 1mM sodium orthovanadate, 1mM sodium molybdate). Cells were solubilised using a cell scraper and the resulting lysates were transferred to 1.5ml plastic tubes and rotated at 4°C for 10 mins. The samples were then centrifuged at 13,000 rpm for 10 mins at 4°C and

the protein containing supernatant was transferred to a fresh eppendorf tube. Samples could be frozen at -80°C at this point.

### **2.3.3. Protein assay**

Total protein per lysate was estimated using the Bio-Rad Protein Assay. This assay is based on the Bradford dye-binding procedure. Known concentrations of bovine serum albumin (BSA) diluted in lysis buffer were used as a standard curve. 5µl of sample was diluted in 1 ml of 20% Bradford solution. 100 µl of Bradford samples was transferred into 96-well plate and read at 595nm on a Dynatech MR5000 platereader. The protein concentrations were calculated by linear regression from the standard curve.

### **2.3.4. Whole cell extracts**

50µl of 5X SDS sample buffer (5% SDS, 50% glycerol, 200mM Tris-HCl pH 6.8, bromophenol blue, 5% 2-mercaptoethanol) was added to each protein sample. Samples were then vortexed briefly, boiled for 5 mins and spun down before loading onto a gel. Samples could be frozen at -80°C at this point.

### **2.3.5. Separation of cellular proteins by electrophoresis**

Proteins were analysed by one dimensional gel electrophoresis, which under reducing conditions separates proteins based on molecule size. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out



essentially as described by Laemmli (1970). Proteins were separated by SDS-PAGE using the Bio-Rad Mini Protean II.

Minigels of the appropriate percentage were prepared as described in buffers and solutions section. The resolving gel was poured into the gel equipment and overlaid with MilliQ water. After 20 mins the resolving gel set and the water was removed. The stacking gel was poured on the top of the running gel and set with a 10 or a 15 lane comb inserted. When the stacking gel was set the comb was removed, the wells were washed thoroughly with MilliQ water followed by running buffer. Then 25 µg of sample was loaded per lane (~10-20µl) alongside a molecular weight marker. Gels were run at room temperature at 80 volts, until the bromophenol blue tracking dye entered the running gel. The voltage was then increased to 180 volts. Gels were run until the bromophenol blue band had reached the bottom of the resolving gel. Gels were then placed into transfer buffer.

#### **2.3.6. Semi-dry transfer of proteins to nitrocellulose**

The graphite electrodes of the semi-dry transfer apparatus (Pharmacia-Biotech Multiphor II) were dampened with semi-dry transfer buffer (39mM glycine, 48mM Trizma base, 0.0375% SDS, 20% (v/v) methanol, MilliQ water). This was followed by placing a sandwich of four pieces of 3MM Whatman paper cut to the size of the gel, one piece of nitrocellulose membrane, the gel and another four pieces of 3MM Whatman paper, all soaked in transfer buffer. Each layer was rolled gently to expel air bubbles. The transfer was run for one hour at 0.8mA/cm<sup>2</sup> of membrane (~40mA

per gel). The membrane was then stained with Ponceau S to check for transfer and equal loading of the samples and also to confirm the location of the molecular weight markers. The stain was removed by washing the membrane in distilled water for 2 mins, followed by a 10 mins wash in Tris buffered saline (TBS; 20mM Tris-HCl pH 7.5, 150mM NaCl, MilliQ water).

### **2.3.7. Immunoblotting of nitrocellulose bound protein**

Non-specific binding sites on the nitrocellulose were blocked by 1 hour incubation of the membrane with the appropriate blocking buffer at room temperature on a rocking platform. The nitrocellulose membrane was washed for 10 mins in TBS-Tween 0.1% and then incubated with the primary antibody made up in TBS-Tween 0.1% overnight. Blocking buffer was added to some primary antibodies; see section 6.2 for specific probing conditions. The membrane was then washed five times for 5 mins each on a rocking platform at room temperature with TBS-Tween 0.1%. The membrane was then incubated for 1 hour with the appropriate secondary antibody diluted in TBS-Tween 0.1% Tween. After this incubation washings were repeated as previously described, before a final wash for 5 mins in TBS. 5ml of Enhanced Chemiluminescent Lumigen (ECL) reagent was added for 1 min and the membrane was then exposed to X-ray film for 2 sec – 30 mins. The film was developed using an RGII Fuji X-ray film developer.

### **2.3.8. Membrane stripping**

Where appropriate, blots were stripped of bound antibody and reprobed with a different primary antibody. After the ECL procedure described above, the

membrane was washed once in TBS for 10 mins and then placed in 50ml of stripping buffer (62.5mM Tris-HCl pH 6.8, 2% (w/v) SDS 100mM 2-mercaptoethanol, MilliQ) water in a sealed sandwich box and incubated at 60°C for 30 mins with regular mixing. After extensive washing in at least three changes of TBS-Tween 0.1.% followed by a TBS wash, the membrane would be reblocked in the appropriate blocking buffer before the addition of the new primary antibody.

#### **2.4. Nuclear extract preparation**

Cells were washed with PBS and 400µl of Buffer 1 (10mM HEPES, 10mM KCl, 100µM EDTA, 100µM EGTA, 1mM sodium molybdate, 1mM sodium vanadate, 10mM sodium fluoride, MilliQ water) was added to cells. Adherent cells were carefully scrapped off the Petri dishes with a rubber scrapper and were transferred into plastic tubes. Cells were put on ice for 15 mins followed by the addition of 15 µl of 10% NP-40 which lyses the cell membrane. Samples were then centrifuged at 13,000g for 5 min to pellet the nuclei. The supernatant, which contains the cytosolic proteins, was transferred to new tubes. The nuclei pellet was then washed in Buffer 1 and resuspended in 50µl Buffer 2 (10mM HEPES, 400mM NaCl, 100µM EDTA, 100µM EGTA, 1mM sodium molybdate, 1mM sodium vanadate, 10mM sodium fluoride). Samples were rotated for 30 mins at 4°C. Finally, the samples were centrifuged at 13,000g for 5 min and the supernatant, containing the nuclear extracts were retained. Bradford assay was carried out to determine protein concentration. Samples could be frozen at -80°C at this point.

Nuclear extracts were utilised for the assessment of transcription factor activation by aid of TransAM NF $\kappa$ B kits (Active Motif, Belgium). 5 X SDS sample buffer was added to an aliquot of both cytosolic and nuclear extracts and samples were boiled for 5 mins before being loaded onto polyacrylamide/sodium dodecyl sulphate gels to determine PPAR $\gamma$  expression or I $\kappa$ B degradation. Western blot analysis was then carried out as described above.

## **2.5. *In vitro* lipid kinase assay**

### **2.5.1. Overview**

PI3K activity can be measured directly by an *in vitro* lipid kinase assay. This assay detects the transfer of radiolabeled  $\gamma$ -phosphate of ATP to the D-3 position of PtdIns, resulting in the formation of ( $^{32}$ P)-labelled PtdIns(3)*P*.

### **2.5.2. Immunoprecipitation and reaction**

Cells were stimulated and lysates were pre-cleared with either 20  $\mu$ l protein A or G sepharose beads for 1 hour at 4°C with constant rotation. Samples were then spun at 13,000g and supernatants were transferred into clean tubes. Proteins were then immunoprecipitated, by the addition of 1 $\mu$ l of the antibody against the protein of interest, and were rotated at 4°C for one hour. 40  $\mu$ l 50% (v/v) suspension of the protein-A or G sepharose beads were then added, and the samples were again rotated for 1 hour at 4°C. The beads were captured by centrifugation at 12,000g for 1 minute at 4°C. The beads were then extensively washed as follows-

3 x in wash buffer A (PBS, 1% NP-40, 100 $\mu$ M sodium vanadate)

3 x in wash buffer B (100mM Tris, pH 7.4, 5mM LiCl, 100 $\mu$ M sodium vanadate)

2 x TNE buffer (10mM tris, pH 7.4, 150mM NaCl, 5mM EDTA, 100 $\mu$ M sodium vanadate).

Between each wash the beads were spun down at 12-14,000g for 15 seconds. After the removal of the final wash pre-treatment with inhibitors (made up in 50  $\mu$ l TNE buffer) was carried out where appropriate. Control samples were treated with 50  $\mu$ l TNE for the same period of time. Following pre-treatment, 10  $\mu$ l (20 $\mu$ g) PI (PtdIns) and 10 $\mu$ l 100mM MgCl<sub>2</sub> were added to each pellet of beads. The reactions were started by adding 5  $\mu$ l of ATP reaction buffer (0.88 mM ATP, 20 mM MgCl<sub>2</sub>, and 5-10  $\mu$ Ci ( $\gamma$ <sup>32</sup>P)-ATP) and were allowed to proceed for 10 minutes. Reactions were terminated by the addition of 20  $\mu$ l 5N HCl and 160 $\mu$ l chloroform/methanol (1:1). Samples were briefly vortexed and phases were separated by centrifuging for 10 min. 50 $\mu$ l of the lower chloroform phase was removed and spotted onto 1% potassium oxalate-treated thin-layer chromatography (TLC) plates. The lipids were resolved by TLC in chloroform:methanol:water:ammonium hydroxide (60:47:11.3:2). (<sup>32</sup>P)-labelled lipids were visualised by autoradiography at -80°C. TLC plates were iodine stained to confirm equal loading.

## 2.6. NFκB Luciferase reporter assay

### 2.6.1. Overview

Genetic reporters are commonly used to study gene expression. Firefly luciferase is widely used as a reporter for the following reasons:

- 1) Reporter activity is available immediately upon translation since the protein does not require post-translation processing.
- 2) The assay is very sensitive
- 3) The assay is rapid, reliable and easy to perform

Firefly luciferase is a 61kDa monomer that catalyzes the mono-oxygenation of beetle luciferin (reaction shown below Figure 2.1.) Beetle luciferin is a relatively stable molecule found only in luminous beetles (which include fireflies). The enzyme uses ATP as a cofactor, although most of the energy for photon production comes from molecular oxygen. The gene encoding firefly luciferase (*luc*) is a cDNA clone that has been incorporated into a number of vectors.

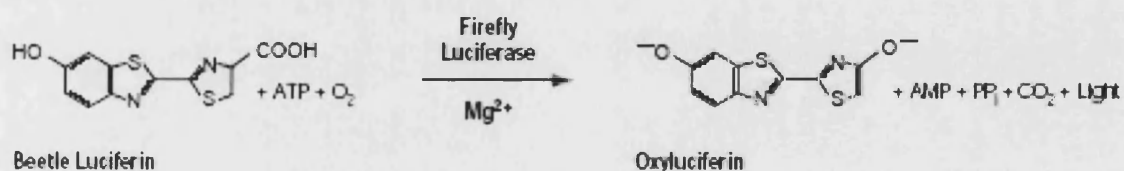


Figure 2.1. Catalyzation of beetle luciferin by firefly luciferase (Promega)

### 2.6.2. Assay procedure

A549-6κBtkluc cell line, obtained from Robert Newton (University of Warwick), is a cell line stably transfected with the NFκB luciferase reporter (Newton et al., 1998). The NFκB-dependent reporter, 6κBtkluc, contains three tandem repeats of the

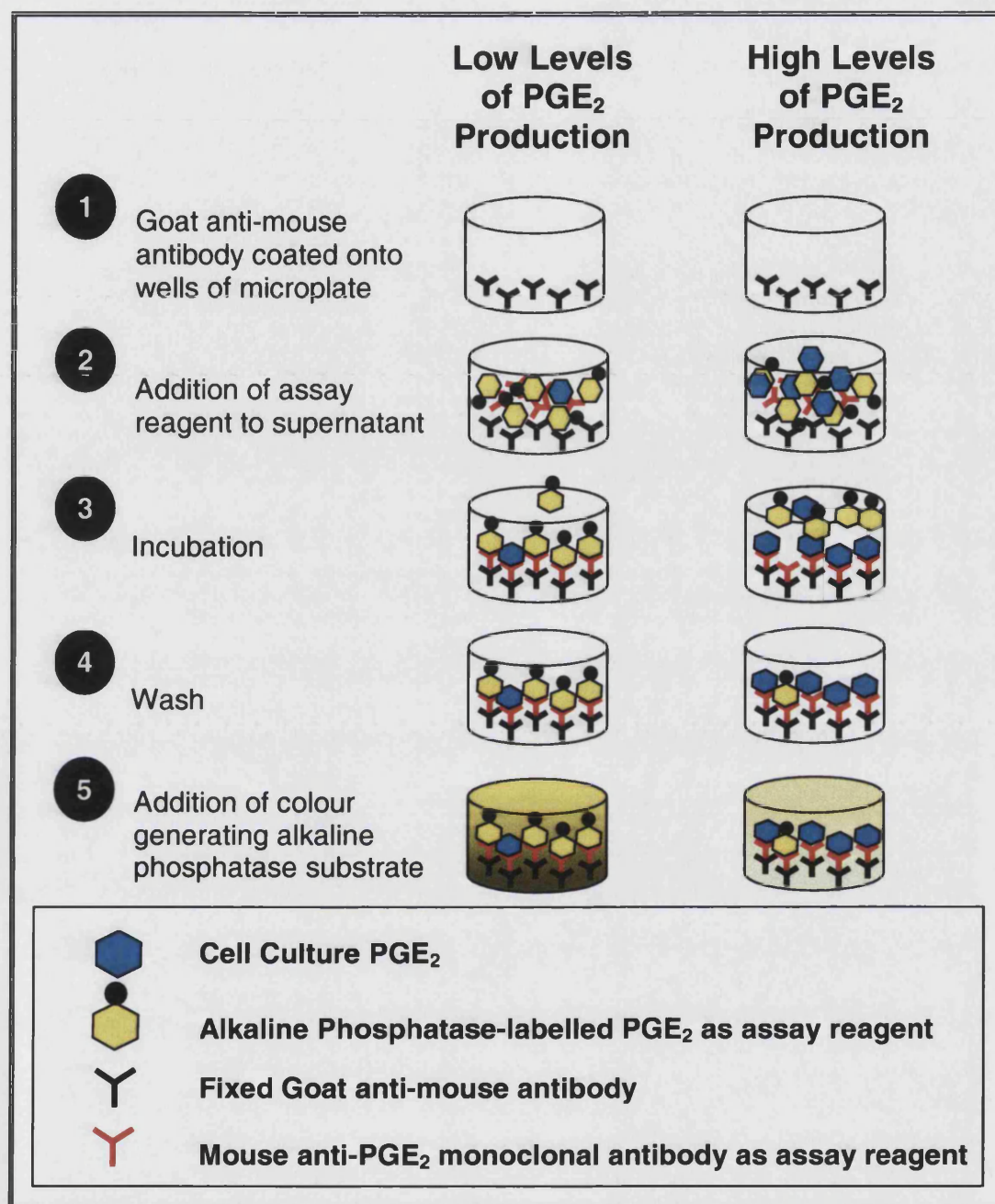
sequence 5'-AGC TTA CAA GGG ACT TTC CGC TGG GGA CTT TCC AGG GA-3', which contains two copies of the decameric NFκB site (underlined), upstream of a minimal thymidine kinase promoter (positions -105 to +51) driving a luciferase gene (Newton et al., 1998). To allow selection of stable transfectants the 6κBtkluc vector was opened with *PvuI* and a *HincII* (blunted- *PvuI* fragment containing the pMC1neoPolyA (Stratagene) neomycin gene was inserted in an opposite orientation to the luciferase gene to generate 6κBtkluc.neo3. The transfection process utilises Tfx50 reagent (Promega) (Newton et al., 1998).

Following cell treatment for the appropriate time with PPARγ ligands, cytokines and/or inhibitors, Promega Steady Glo buffer/substrate (200 μl) was added to each well, mixed thoroughly, and left to stand at room temperature for 10 mins. The supernatant was mixed further and 200 μl from each well was transferred to an opaque-walled 96-well plate (Costar). Luminescence was measure on a Beckman Topcounter with a 5 sec read per well.

## **2.7. Prostaglandin E<sub>2</sub> ELISA**

### **2.7.1. Overview**

This assay to measure PGE<sub>2</sub> concentration uses a kit from R&D Systems (Abingdon, UK). It is based on the competitive binding technique in which PGE<sub>2</sub> present in the sample competes with a fixed amount of alkaline phosphatase-labelled PGE<sub>2</sub> for sites on a mouse monoclonal antibody (Figure 2.2).



**Figure 2.2 Schematic representation of prostaglandin ELISAs.** This demonstrates the competitive binding technique used in the ELISAs for PGE<sub>2</sub> in this work. See text for full explanation

During the incubation, the mouse monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to



determine the bound enzyme activity. The intensity of the colour generated is inversely proportional to the concentration of PGE<sub>2</sub> in the sample.

### **2.7.2. Assay procedure**

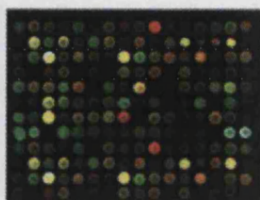
Known concentrations of a PGE<sub>2</sub> standard were diluted in cell culture medium and used for a standard curve (39 pg/ml to 5000 pg/ml). 100 µl of standard or diluted (1:5) cell culture supernatant sample were placed in microplates coated with goat anti-mouse antibody. To each was added 50 µl of PGE<sub>2</sub> conjugate (conjugated to alkaline phosphatase) and 50 µl of PGE<sub>2</sub> antibody solution (mouse monoclonal). The plates were covered and incubated on a shaking platform for two hours at room temperature. After this incubation the wells were aspirated and washed with 200 µl wash buffer using a multi-channel pipette. This was repeated to give a total of three washes and after the last wash the microplates were inverted and blotted against clean paper towelling. 200 µl of pNPP substrate was then added to all wells and incubated at room temperature without agitation. The optical density at 405nm was determined immediately using a Dynatech MR5000 plate reader. The PGE<sub>2</sub> concentrations were calculated by comparison to the sigmoid standard curve using BioLinx software and dilution factors were taken into consideration.

## **2.8. Microarray**

### **2.8.1. Overview**

Microarrays can be used to analyse the expression of tens of thousands of genes simultaneously. Microarrays are miniature devices containing thousands of DNA

sequences stuck on at different positions (addresses). Hybridization to complex mixtures of labelled DNA molecules, prepared from cellular RNA, shows the relative expression levels of thousands of genes (see below Figure 2.3). This can be used to compare gene expression levels within a sample or look at differences in the expression of specific genes across different samples.



**Figure 2.3.** Gene chip showing relative expression levels

### **2.8.2. RNA isolation**

RNA was isolated from A549 cells using Trizol following cell stimulation in 10 cm<sup>2</sup> petri dishes. After the removal of cell culture supernatant, 2 ml of Trizol was added to the cells for 1 minute, resulting in cell lysis. The sample was solubilised by passing the lysate through a pipette several times. The samples were then placed in two 1.5 ml plastic tubes and frozen at -80°C. When thawed 200 µl of chloroform was added to each tube and the samples were shaken vigorously for 15 seconds. They were then incubated at room temperature for 3 minutes before being centrifuged at 12,000g for 15 mins at 4°C. The DNA and proteins were in the interphase and organic phase while the aqueous phase contained the RNA which was carefully pipetted off into a new eppendorf. 500 µl of isopropanol was then added to the aqueous phase and the samples stored at room temperature for 10 mins. They were then centrifuged at 12,000g for 10 mins at 4°C and an RNA pellet was subsequently visible at the bottom

of the tube. The supernatant was removed and the pellet washed with 1ml cold 75% ethanol, centrifuged at 7500g for 5 mins at 4°C. The supernatant was removed and the pellet was air-dried. Each pellet was resuspended in 45 µl of RQI buffer and the RNA from both pellets combined. RNA samples were incubated at 37°C for 30 mins, put through QIAGEN RNeasy columns and eluted in 30 µl RNase-free water. RNA was then quantified and 100ng per well was run on RNA chips on Agilent 2100 Bioanalyzer.

RNA samples were processed for hybridisation on Affymetrix HG U133A (Affymetrix Inc, Santa Clara, US) gene chip by the Genomics Factory EU (Novartis), following standard procedures. The Gene chip Human Expression Array HG U133A contains approximately 22,000 probe sets representing more than 20,000 transcripts derived from approximately 17,000 well substantiated human genes. This design uses sequences selected from GenBank, dbEST and RefSeq. The sequence clusters were created from the Unigene database (Build 133, April 20, 2001).

### **2.8.3. Microarray analysis**

Data analysis was performed either in Genespring 5 (Silicon Genetics, Redwood city, US) or Excel (Microsoft). The default normalisation, recommended by Genespring was applied. It consisted of two steps (1) per chip normalisation (global normalisation) and (2) per gene normalisation. This resulted in transforming Affymetrix MAS5 gene expression data to a normalised constant value of 1 in Genespring. The genes were subsequently filtered by setting a minimal expression value (the smallest value any gene is allowed to have, here 20) to be met in at least

50% of the conditions. This restriction was applied to raw data. The reason for this trimming was to discount gene expression values which can not be distinguished from background noise, and therefore may account for false positives.

## **2.9. TransAm NF $\kappa$ B kit**

### **2.9.1. Overview**

TransAM™ method, developed by Active Motif, introduced the first ELISA-based kits to detect and quantify transcription factor activation. TransAM NF $\kappa$ B kits contain a 96-well plate to which oligonucleotide containing the NF $\kappa$ B consensus binding site (5'GGGACTTCC-3'), have been immobilized. The active form of NF $\kappa$ B p65 contained in cell extract specifically binds to this oligonucleotide. The primary antibody used to detect NF $\kappa$ B recognizes an epitope on p65 that is accessible only when NF $\kappa$ B p65 is activated and bound to its target DNA. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provides sensitive colorimetric readout that can be quantified by spectrophotometry. Figure 2.4 outlines the assay procedure.

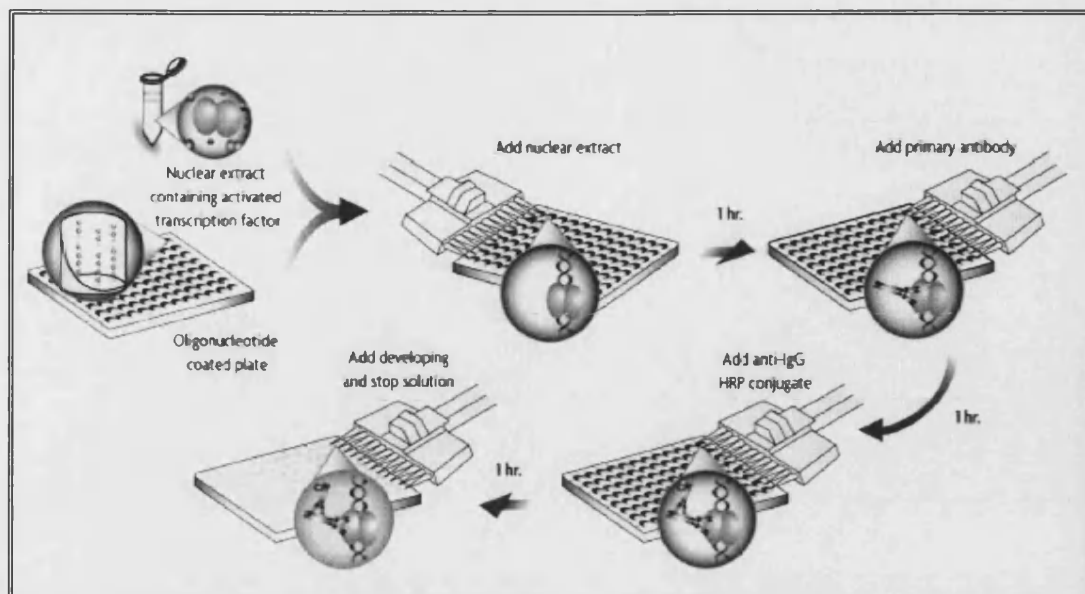


Figure 2.4. Outline of the TransAM NF $\kappa$ B assay procedure

### 2.9.2. Assay procedure

Sample (20  $\mu$ l; containing 7.5-10  $\mu$ g protein) was diluted in complete lysis buffer was added in duplicate to wells containing 30  $\mu$ l binding buffer. Appropriate blank and positive controls were carried out. The plate was then covered and incubated at room temperature with mild agitation for one hour. The wells were washed 3 times with 200  $\mu$ l of 1X washing buffer. For each wash, the plate was flicked over a sink to empty wells, and then the inverted plate was trapped three times on absorbent paper towels. Diluted p65 NF $\kappa$ B antibody (100  $\mu$ l; 1:1000 dilution in 1X antibody binding buffer) was added to wells of the TransAM NF $\kappa$ B kit. The plate was cover and incubated for one hour at room temperature without aggitiation, followed by three washes as described above. 100  $\mu$ l diluted anti-rabbit (1:1000 dilution in 1X antibody binding buffer) was then added to each well. The plate was covered and

further incubated for one hour at room temperature without agitation, followed by four washes as described above. 100 µl of room-temperature developing solution was added to wells and incubated for 10 minutes protected from direct light. Finally a 100 µl of stop solution was added and the absorbance on a spectrophotometer was read at 450 nm.

## **2.10. Statistics**

One way ANOVA test with Bonferroni Correction was carried out to test for statistical significance of ELISA and NFκB activation data. A statistical cut-off of a P value of 0.05 was used. In order to derive differentially expressed genes from the microarray analysis, a Welch t-test (parametric, not assuming equal variances) was used with a statistical cut-off of a P value of 0.05. No multiple testing corrections were applied and fold change filtering was also performed in Genespring. The average expression value per gene from three separate experiments was used to define the fold change as a ratio. The fold regulation cut-off used was a >2 fold up or downregulation.



**2.11. Materials**

Material	Source
( <sup>32</sup> P)γ-ATP	Amersham International, (UK)
2-Mercaptoethanol	Sigma, Poole (UK)
15d-PGJ <sub>2</sub>	Calbiochem (UK)
Absolute Ethanol	BDH, Poole (UK)
Acrylamide/bis acrylamide	Bio-Rad, (UK)
Adenosine triphosphate	Sigma, Poole (UK)
Ammonium persulphate	BDH, Poole (UK)
BADGE	Tocris, Avonmouth (UK)
Bovine serum albumin (BSA)	Sigma, Poole (UK)
Bromophenol blue	BDH, Poole (UK)
Cell culture plastics	Nunc, (UK)
Chloroform	Fisons, Loughborough (UK)
Anti-COX-1 Ab (C-20; sc-1752)	Santa Cruz, (USA)
Anti-COX-2 Ab	Alexis (Nottingham, U.K.).
Chloroform	Fisons, Loughborough (UK)
Dimethyl sulphoxide (DMSO)	Sigma, Poole (UK)
Dulbecco's Modified Eagle Medium	Gibco BRL, Paisley (UK)
Enhanced chemiluminescence detection kit for Western blotting (ECL)	Amersham International, (UK)
Anti-ERK1/2 polyclonal Ab (sc-93)	Santa Cruz, CA (USA)
Anti-ERK1/2 <sup>Thr202/Tyr204</sup> polyclonal Ab (cat. no. 9101)	Cell signalling (UK)
Ethylenediaminetetraacetic acid (EDTA)	Sigma, Poole (UK)
Foetal bovine serum (FBS)	Gibco BRL, Paisley, (UK)
Formaldehyde	BDH, Poole (UK)
Glycerol	Sigma, Poole (UK)
Glycine	Sigma, Poole (UK)
Goat anti-rabbit peroxidase conjugate	DAKO, Glostrup, Denmark
GW9662	Tocris, Avonmouth (UK)

HEPES (1M liquid)	Gibco BRL, Paisley, (UK)
Hydrochloric acid	BDH, Poole (UK)
Anti-IκB Ab (C-15; sc-203)	Santa Cruz, CA (USA)
IL-1α: human recombinant; specific activity 5×10 <sup>7</sup> U/mg	Peprotech, London (UK)
L-glutamine	Gibco BRL, Paisley, (UK)
Leupeptin	Sigma, Poole (UK)
Lithium chloride (LiCl)	Sigma, Poole (UK)
LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one)	Tocris, Avonmouth (UK)
Methanol	Fisons, Loughborough (UK)
Molecular weight markers	Bio-Rad, Hertfordshire (UK)
Anti-mPGES polyclonal Ab	Alexis (Nottingham, U.K.).
Anti-NFκB p65- <sup>276</sup> Ser polyclonal Ab (cat. no. 3037)	Cell signalling, Hitchin, (UK)
Nitrocellulose	BDH (UK)
NP40	Sigma, Poole (UK)
Anti-PI3K-p110 Ab (sc-7177)	Santa cruz, CA (USA)
Anti-PI3K-p85 Ab	Gift from J. Domin, (Imperial College, London, U.K)
PD98059	Alexis (Nottingham, U.K.)
Penicillin/ Streptomycin	Gibco BRL, Paisley, (UK)
PGE <sub>2</sub> ELISA kit	R & D Abingdon (UK)
Phosphate buffered saline	Gibco BRL, Paisley, UK
Phosphatidylinositol	Sigma, Poole (UK)
Anti-PI3K- C2α Ab	Gift from J. Domin (Imperial College, London, U.K)
Anti-PI3K- C2β Ab	Gift from J. Domin (Imperial College, London, U.K)
Anti-PKB-α <sup>473</sup> Ser, polyclonal Ab (cat. no. 9271)	New England Biolabs, USA
Anti-PKB-α, polyclonal Ab (sc-1618)	Santa Cruz, CA (USA)



PMSF	Sigma, Poole (UK)
Ponceau S	Sigma, Poole (UK)
Anti-PPAR $\gamma$ polyclonal Ab	Alexis (Nottingham, U.K.)
Promega Steady Glo luciferase buffer	Promega, Southampton, (UK)
Propan-1-ol	Fisons, Loughborough (UK)
Propan-2-ol	Fisons, Loughborough (UK)
Protein G beads	Amersham (UK)
Ro-32-0432	Alexis (Nottingham, U.K.)
RPMI 1640	Gibco BRL, Paisley, (UK)
Sodium azide	Sigma, Poole (UK)
Sodium chloride	Sigma, Poole (UK)
Sodium dodecyl sulfate (SDS)	Sigma, Poole (UK)
Sodium fluoride	Sigma, Poole (UK)
Sodium hydroxide	Sigma, Poole (UK)
Sodium molybdate	Sigma, Poole (UK)
Sodium orthovanadate	Sigma, Poole (UK)
TEMED	Sigma, Poole (UK)
Thin layer chromatography plates	Whatmann (UK)
Tissue culture reagents	Gibco BRL, Paisley, UK
TNF- $\alpha$ : human recombinant; specific activity $6 \times 10^7$ U/mg	Peprtech, London (UK)
TransAM <sup>TM</sup> NF $\kappa$ B kits	Actif Motif, Rixensart, Belgium
Trizma base	Sigma, Poole (UK)
Troglitazone	Alexis (Nottingham, U.K.)
Trypan Blue	Sigma, Poole (UK)
Trypsin EDTA	Gibco BRL, Paisley, (UK)
Tween-20	Sigma, Poole (UK)
Wortmannin	Sigma, Poole (UK)
X-OMAT film	Amersham International, UK

# **3.**

## **RESULTS I**

### **3. RESULTS I**

#### **3.1. Selectivity of PI3K inhibitors to Class IA and Class II PI3K**

##### **3.1.1. Background**

This thesis investigates the role of the PI3K pathway in regulation of COX-2 expression by cytokines and PPAR $\gamma$  ligands in A549 cell line. In the first section of this chapter the involvement of the PI3K pathway was evaluated by the use of two distinct pharmacological inhibitors of PI3K, LY294002 and wortmannin. Wortmannin is derived from a metabolite of the fungus *Penicillium fumiculosum thom* and LY294002 is derived from quercetin.

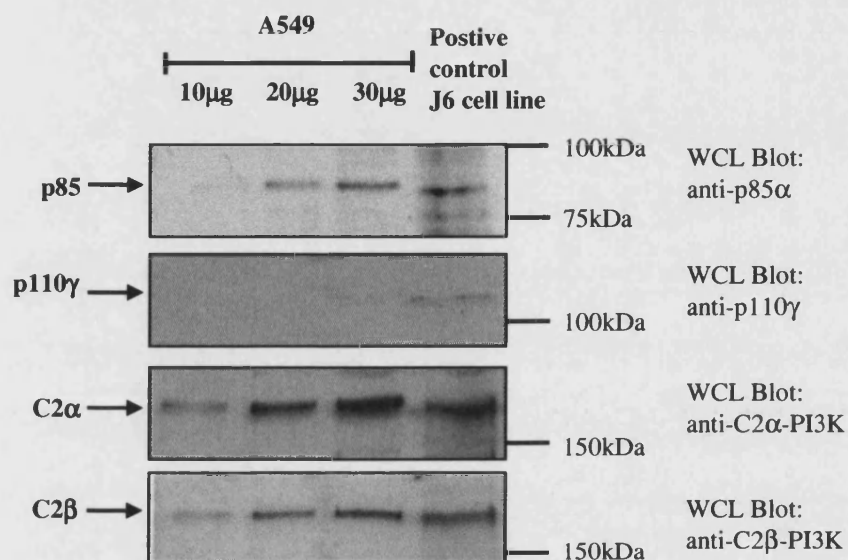
Previous work has shown that LY294002 and wortmannin are poor inhibitors of recombinant PI3K-C2 $\alpha$  (Domin et al., 1997). Thus the selectivity of LY294002 and wortmannin to PI3K isoforms was investigated by *in vitro* lipid kinase assays. The well characterised Jurkat cell line, which have high basal PI3K activity due to the lack of lipid phosphatases SHIP and PTEN, was initially utilised as a positive control and have the additional advantage that they grow rapidly and therefore supply a large number of cells which is required in order to carry out *in vitro* lipid kinase assays.

### **3.1.2. Results**

#### **Expression of PI3K isoforms**

It was necessary to first identify the expression of different isoforms of PI3K in A549 cell line, in order to evaluate which isoforms may be involved in PPAR $\gamma$  mediated signal transduction (investigated in the next chapter). Jurkat whole cell extract was used as a positive control since they have been shown to express Class 1A p85 regulatory subunit. This is known to be tightly coupled to p110  $\alpha$ ,  $\beta$  and  $\delta$  catalytic isoforms which are not thought to exist on their own in the cytoplasm. Hence, p85 $\alpha$  is an indirect maker of p110 catalytic subunit expression; although it does not tell us which Class IA p110 isoforms are expressed. Jurkat cells also express Class 1B PI3K, PI3K-C2 $\alpha$  and PI3K-C2 $\beta$ .

A549 cells showed increasing expression of the regulatory p85 domain of Class IA PI3K isoforms, as well as  $\alpha$  and  $\beta$  isoforms of the Class II PI3Ks (Figure 3.1) when increasing amounts of protein were loaded onto the SDS-page gel. As expected, these cells did not express the Class IB PI3K catalytic subunit p110 $\gamma$ , which has expression predominantly restricted to cells of haematopoietic lineage (Vanhaesebroeck and Waterfield, 1999). Expression of all four isoforms investigated were seen in the Jurkat positive control.



**Figure 3.1** The A549 cell line express PI3K isoforms.

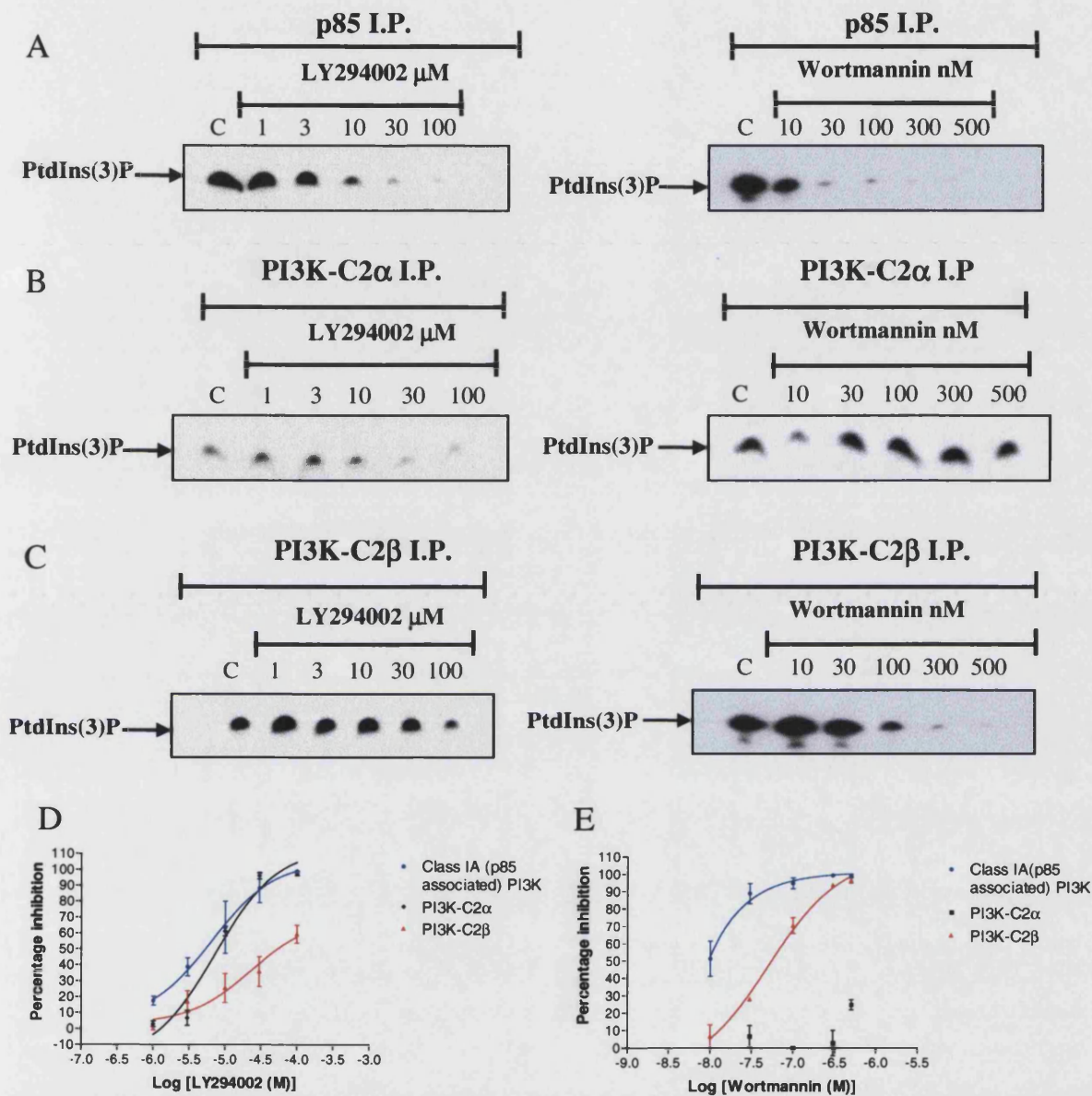
$3.2 \times 10^6$  grow-arrested, confluent A549 cells were lysed with lysis buffer to obtain whole cell lysates (WCL). Positive control lysates whole cell lysate of Jurkat (J6) cell line for PI3K isoforms were also collected. Proteins were quantified by Bradford assay and boiled with  $5 \times$  sample buffer, resolved by SDS-PAGE (20μg of protein was loaded for the positive control), transferred to nitrocellulose membranes and immunoblotted, as described in *Methods and Materials*, with Ab raised against specific PI3K isoforms (B; anti-p85, anti-p110γ, PI3K-C2α and PI3K-C2β). Proteins were visualized with ECL. Data is representative of three experiments.

### **Effect of PI3K inhibitors on Class IA and Class II PI3K isoforms in Jurkat cell line**

Anti-p85 antibody was used to immunoprecipitate Class IA PI3K catalytic subunits which are associated with the regulatory p85 subunit. Anti-PI3K-C2 $\alpha$  antibody was utilised to immunoprecipitate Class II $\alpha$  PI3K and similarly anti-PI3K-C2 $\beta$  antibody was used to immunoprecipitate Class II $\beta$  PI3K isoform.

The activities of Class IA PI3K, PI3K-C2 $\alpha$  and PI3K-C2 $\beta$  were sensitive to LY294002 (Figure 3.2A, 3.2B and 3.2C respectively; left panels) although at varying degrees in the Jurkat cell line. In comparison, wortmannin inhibited Class IA PI3K and PI3K-C2 $\beta$  (Figure 3.2A and 3.2C respectively; right panels), but had little effect on PI3K-C2 $\alpha$  activity (Figure 3.2B; right panel). Densitometry was carried out and utilised to calculate IC<sub>50</sub> values of the inhibitors to PI3K activity. The rank order of sensitivity of LY294002 was Class IA PI3K (IC<sub>50</sub>= 6.5  $\mu$ M) > PI3K-C2 $\alpha$  (IC<sub>50</sub>= 7.3  $\mu$ M) > PI3K-C2 $\beta$  (IC<sub>50</sub>=23.1  $\mu$ M) (calculated from Figure 3.2D). Whereas, the rank order of sensitivity of wortmannin was Class IA PI3K > (IC<sub>50</sub>= 8.7 nM) > PI3K-C2 $\beta$  (IC<sub>50</sub>=51.2 nM)>PI3K-C2 $\alpha$  (IC<sub>50</sub>= > 500 nM) (calculated from Figure 3.2E).



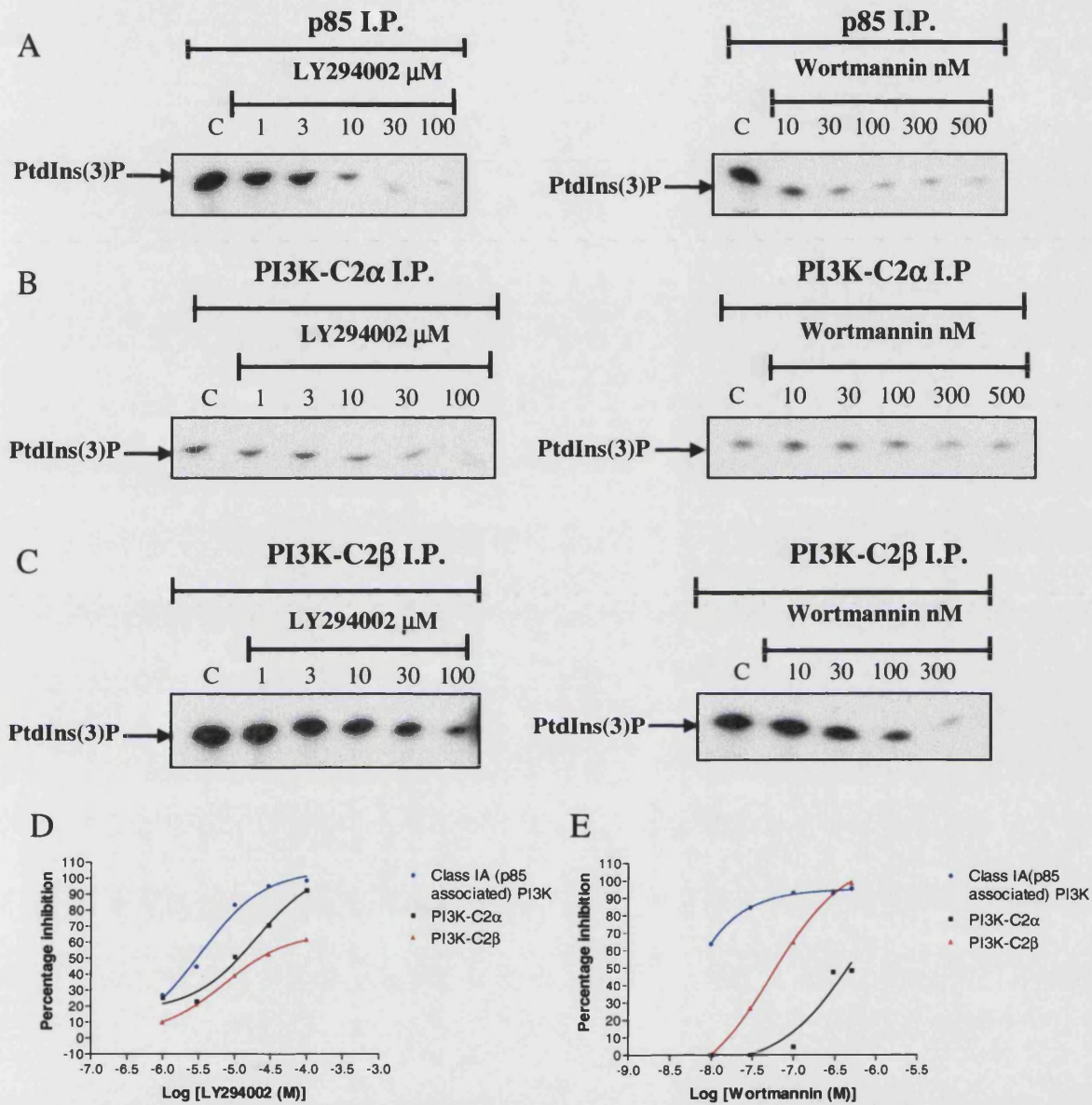


**Figure 3.2 Sensitivity of PI3K isoforms to LY294002 and Wortmannin in Jurkat cell line.** Whole cell lysates from unstimulated cells were immunoprecipitated (I.P.) for Class IA-p85 associated PI3K (A), PI3K-C2 $\alpha$  (B), and PI3K-C2 $\beta$  (C) and assayed for *in vitro* lipid kinase activity in the presence of increasing concentration of LY294002 (pre-treatment for 30mins prior to initiation of reaction), as indicated (left hand panels) or pre-treated Wortmannin for 10 mins, as indicated (right hand panels). Reactions were carried out in the presence of  $Mg^{2+}$  buffer. Reaction products were extracted, fractionated by thin layer chromatography, and examined by autoradiography. Data is representative of three experiments. Results were quantified by densitometry, a dose response curve created and  $IC_{50}$  values calculated (D and E). Error bars illustrate standard deviation.

**Effect of PI3K inhibitors on Class IA and Class II PI3K isoforms in A549 cell line**

Next it was investigated whether the same differential sensitivity of the PI3K inhibitors was also seen in A549 cell line. As demonstrated in Jurkat cell line, the activities of Class IA PI3K, PI3K-C2 $\alpha$  and PI3K-C2 $\beta$  were sensitive to LY294002 (Figure 3.3A, 3.3B and 3.3C respectively; left panels) although at varying degrees in A549 cell line. In comparison, wortmannin inhibited Class IA PI3K and PI3K-C2 $\beta$  (Figure 3.3A and 3.3C respectively; right panels), but had little effect on PI3K-C2 $\alpha$  activity (Figure 3.3B; right panel). Again, densitometry was carried out and utilised to calculate IC<sub>50</sub> values of the inhibitors to PI3K activity. The rank order of sensitivity of LY294002 was Class IA PI3K (IC<sub>50</sub>= 3.42  $\mu$ M) > PI3K-C2 $\alpha$  (IC<sub>50</sub>= 7.36  $\mu$ M) > PI3K-C2 $\beta$  (IC<sub>50</sub>= 19.69  $\mu$ M) (calculated from Figure 3.3D). Whereas, the rank order of sensitivity of wortmannin was Class IA PI3K > (IC<sub>50</sub>= 0.219 nM) > PI3K-C2 $\beta$  (IC<sub>50</sub>= 55.73 nM) PI3K-C2 $\alpha$  (IC<sub>50</sub>= > 500 nM) (calculated from Figure 3.3E).





**Figure 3.3 Sensitivity of PI3K isoforms to LY294002 and Wortmannin in A549 cell line.** Whole cell lysates from unstimulated cells were immunoprecipitated (I.P.) for Class 1A-p85 associated PI3K (A), PI3K-C2 $\alpha$  (B), and PI3K-C2 $\beta$  (C) and assayed for *in vitro* lipid kinase activity in the presence of increasing concentration of LY294002 (pre-treatment for 30mins prior to initiation of reaction), as indicated (left hand panels) or pre-treated Wortmannin for 10 mins, as indicated (right hand panels). Reactions were carried out in the presence of Mg<sup>2+</sup> buffer. Reaction products were extracted, fractionated by thin layer chromatography, and examined by autoradiography. Data is representative of three experiments. Results were quantified by densitometry, a dose response curve created and IC<sub>50</sub> values calculated (D and E).

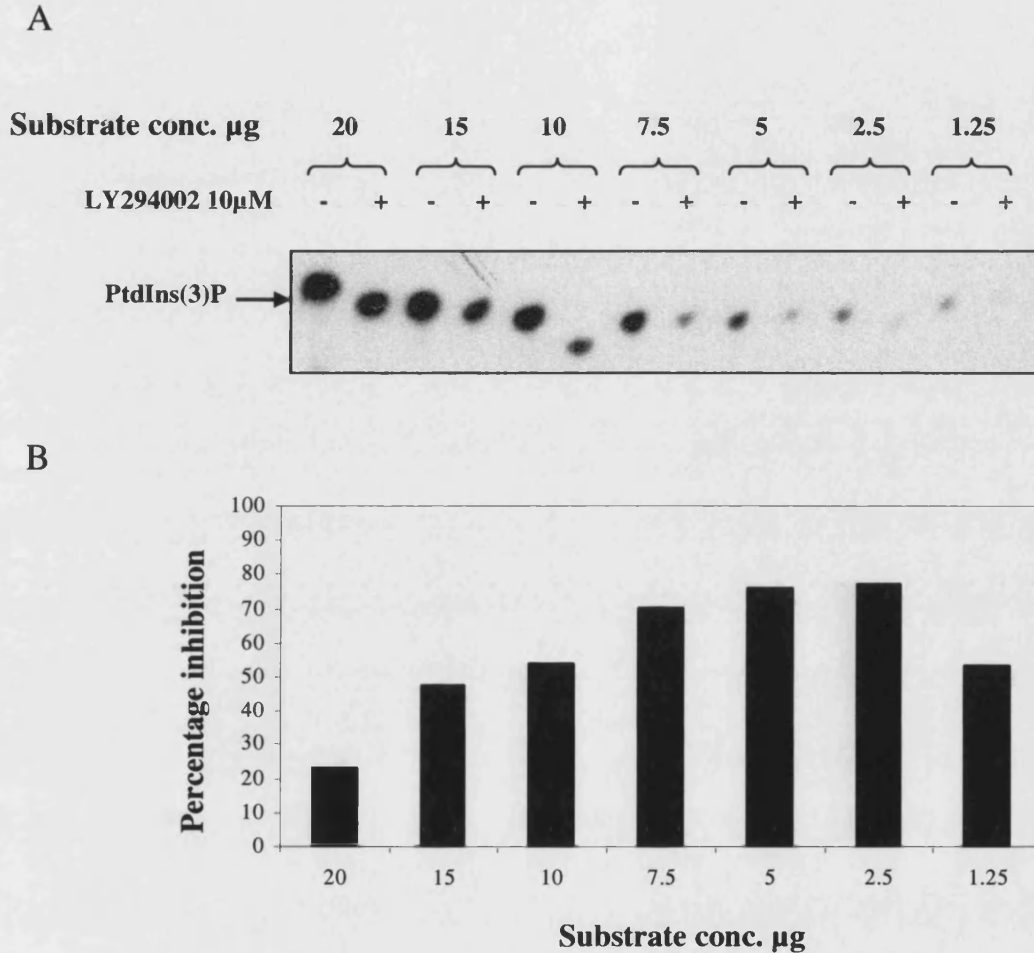
The table below outlines the differential sensitivity of LY294002 and wortmannin to PI3K isoforms in both Jurkat and A549 cell lines.

	LY294002 ( $\mu\text{M}$ )		WORTMANNIN (nM)	
	<u>Jurkat</u>	<u>A549</u>	<u>Jurkat</u>	<u>A549</u>
<b>Class IA PI3K</b>	6.5	3.4	8.7	0.22
<b>PI3K-C2<math>\alpha</math></b>	7.3	7.4	>500	>500
<b>PI3K-C2<math>\beta</math></b>	23.1	19.7	51.2	55.7

**Table 3.1 Comparison of rank order of sensitivity of LY294002 and wortmannin in Jurkat and A549 cell lines** (Data calculated from Figure 4.2 and 4.3; n =3).

#### **Effect of varying amount of substrate (PI) on the sensitivity of LY294002 to PI3K-C2 $\beta$ isoforms**

The amount of substrate (PI) used in *in vitro* lipid kinase assays is thought to play an important role in enzyme kinetics and thus may affect the sensitivity of inhibitors. Thus I investigated whether the sensitivity of LY294002 to PI3K-C2 $\beta$  could be increased by altering substrate concentration. Interestingly, decreasing the amount of substrate (down from 20  $\mu\text{g}$  to 2.5  $\mu\text{g}$ ) increased the sensitivity of LY294002 for PI3K-C2 $\beta$  (from 23.1% inhibition to 77.2%; Figure 3.4A and B).



**Figure 3.4 Sensitivity of PI3K-C2 $\beta$  isoform to LY294002 in the presence of decreasing concentration of substrate in the A549 cell line.** Whole cell lysates from unstimulated cells were immunoprecipitated for PI3K-C2 $\beta$  (A) and assayed for *in vitro* lipid kinase activity in the presence or absence of LY294002 10 $\mu\text{M}$  (pre-treatment for 30mins prior to initiation of reaction), in decreasing amounts of substrate as indicated. Reactions were carried out in the presence of  $\text{Mg}^{2+}$  buffer. Reaction products were extracted, fractionated by thin layer chromatography, and examined by autoradiography. Data is representative of three experiments. Results were quantified by densitometry and the percentage inhibition of LY294002 compared to its respective control are illustrated in (B).

## **3.2. Regulation of TNF $\alpha$ and IL-1 $\beta$ stimulated signalling and COX-2 expression and activity in A549 cell line**

### **3.2.1 Background**

TNF $\alpha$  has previously been shown, in a colonic epithelial cell line, to activate both the PI3K-dependent signalling pathway and the MAPK signalling pathway, which in turn plays key roles in the regulation of COX-2 expression (Weaver et al., 2001). In addition the pro-inflammatory cytokine IL-1 $\beta$  has also been shown to induce COX-2 expression via these signalling pathways in canine tracheal smooth muscle cells (Yang et al., 2002). Thus, the regulatory role of PI3K and MAPK in TNF $\alpha$  and IL-1 $\beta$  induced COX-2 expression in the human lung epithelial cell line A549 was investigated in this latter section of this chapter. This was of importance for further studies, in the next chapter, which investigate the effects of PPAR $\gamma$  ligands on cytokine induced expression of pro-inflammatory mediators such as COX-2.

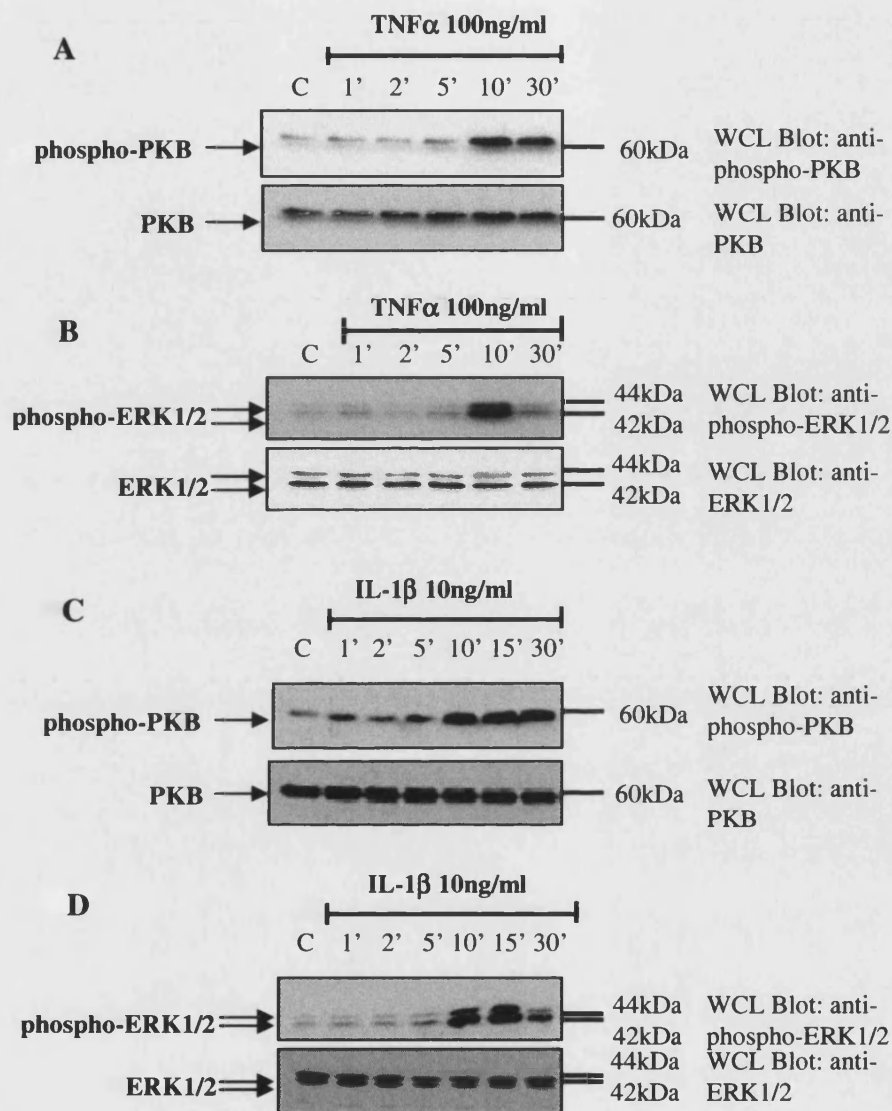
### **3.2.2 Results**

#### **TNF $\alpha$ and IL-1 $\beta$ activate the PI3K and MAPK pathways**

The recruitment, phosphorylation and subsequent activation of PKB are fundamentally dependent on the accumulation of lipid products of PI3K (Vanhaesebroeck and Alessi, 2000) thus phosphorylation of PKB was used as an indirect marker of PI3K activation. Phosphorylation of ERK was used as a marker of activation of the ERK MAPK pathway. TNF $\alpha$  (100 ng/ml) markedly increased PKB

phosphorylation at 10-30 mins (Figure 3.5A), and ERK phosphorylation at 10 mins (Figure 3.5B). Similarly, IL-1 $\beta$  induced activation of PKB (Figure 3.5C) and ERK (Figure 3.5D) at 10-30 mins.

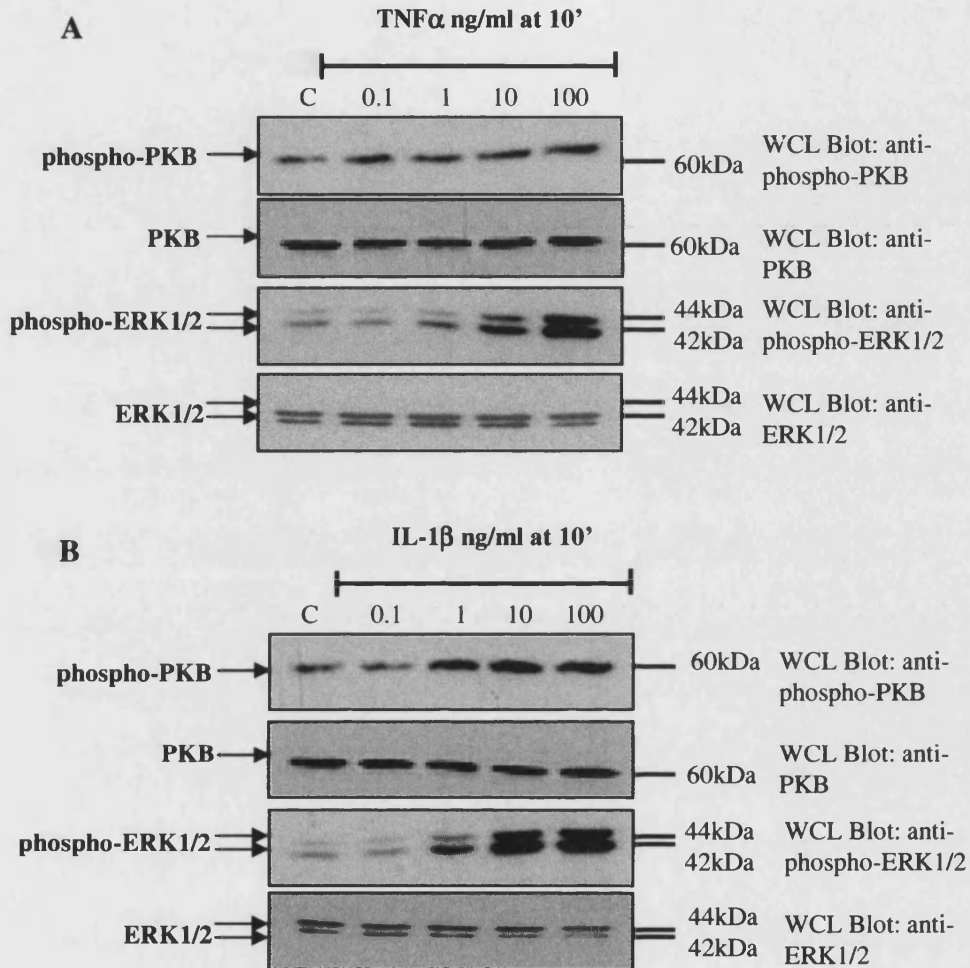
Having determined that TNF $\alpha$  and IL-1 $\beta$  can activate both PKB and ERK MAPK in a time-dependent manner, the effects of increasing concentrations of these cytokines was investigated in order to determine the optimum concentration for further experiments. TNF $\alpha$  induced concentration dependent phosphorylation of ERK, however a similar effect on PKB phosphorylation was not so evident (Figure 3.6A). This may be due to the high basal level of phospho-PKB sometimes seen in A549 cells. IL-1 $\beta$  induced concentration dependent phosphorylation of PKB and ERK (Figure 3.6A and 3.6B respectively). Maximum phosphorylation of ERK with TNF $\alpha$  was observed at 100 ng/ml, whereas with IL-1 $\beta$  it was seen at 10 ng/ml and thus these concentrations were used for further experiments.



**Figure 3.5 Effect of TNF $\alpha$  and IL-1 $\beta$  on PKB and ERK phosphorylation**

$1.2 \times 10^6$  growth arrested, confluent A549 cells were stimulated at 37°C (for the indicated time points) with TNF $\alpha$  (100 ng/ml) (A) or IL-1 $\beta$  (10ng/ml) (B). Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25  $\mu$ g) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted, as described in *Methods and Materials*, with a phospho-specific PKB Ab with affinity for the active Ser<sup>473</sup>-phosphorylated form of PKB (upper panels A and C) or a phospho-specific ERK Ab with an affinity for phosphorylated Thr<sup>202</sup>/Tyr<sup>204</sup> sites (upper panel B and D). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-PKB or anti-ERK Ab to verify equal loading and efficiency of protein transfer (lower panels). Data is representative of three separate experiments.



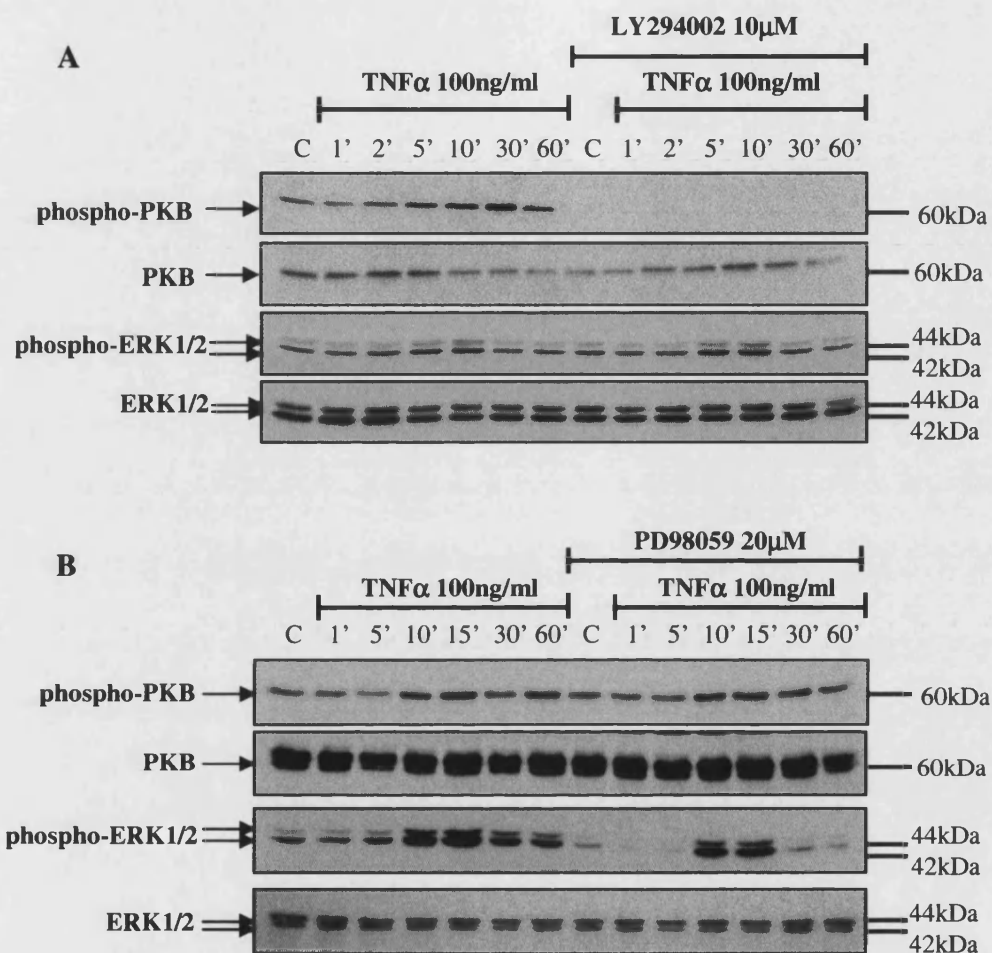


**Figure 3.6** Effect of increasing concentration of TNF $\alpha$  and IL-1 $\beta$  on PKB and ERK phosphorylation.

$1.2 \times 10^6$  growth arrested, confluent A549 cells were stimulated at 37°C (for 10 mins) with increasing concentrations of TNF $\alpha$  (A) or IL-1 $\beta$  (B). Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25  $\mu$ g) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted, as described in *Methods and Materials*, with a phospho-specific PKB Ab with affinity for the active Ser<sup>473</sup>-phosphorylated form of PKB or a phospho-specific ERK Ab with an affinity for phosphorylated Thr<sup>202</sup>/Tyr<sup>204</sup> sites. Proteins were visualized with ECL. Blots were stripped and reprobed with anti-PKB or anti-ERK Ab to verify equal loading and efficiency of protein transfer. Data is representative of three separate experiments.

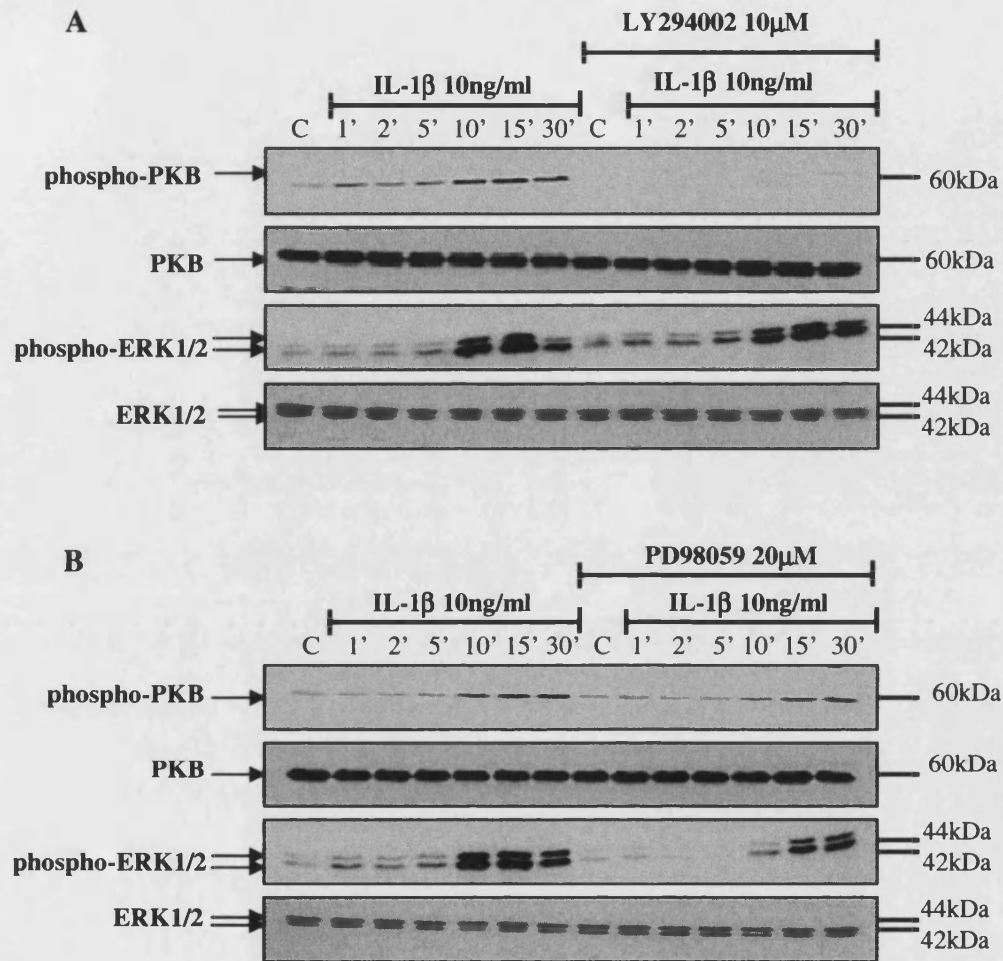
Next the effect of the PI3K inhibitor-LY294002 and the MEK inhibitor PD98059, on TNF $\alpha$ - and IL-1 $\beta$ - induced PKB and ERK phosphorylation were examined (Figure 3.7 and 3.8 respectively). LY294002 (10  $\mu$ M) completely abolished TNF $\alpha$  induced phosphorylation of PKB, but had no effect on TNF $\alpha$  induced ERK phosphorylation at the concentration used (Figure 3.7A). Pretreatment with PD98059 (20  $\mu$ M; an inhibitor of MEK) had no inhibitory effects on TNF $\alpha$  induced PKB phosphorylation, and surprisingly it only modestly inhibited TNF $\alpha$  induced ERK phosphorylation (Figure 3.7B). Similar effects on IL-1 $\beta$ - induced PKB and ERK phosphorylation were seen in the presence of these inhibitors (Figure 3.8A and 3.8B).





**Figure 3.7** Effect of PI3K and MEK inhibitors on TNFα induced signalling events

$1.2 \times 10^6$  growth arrested, confluent A549 cells were pre-treated with LY294002 (10 μM) for 30 mins (A) or with PD98059 (20 μM) for 1 hour (B) prior to stimulation at 37°C (for the indicated time points) with TNFα (100 ng/ml). Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25 μg) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted, as described in *Methods and Materials*, with a phospho-specific PKB Ab with affinity for the active Ser<sup>473</sup>-phosphorylated form of PKB or a phospho-specific ERK Ab with an affinity for phosphorylated Thr<sup>202</sup>/Tyr<sup>204</sup> sites. Proteins were visualized with ECL. Blots were stripped and re-probed with anti-PKB or anti-ERK Ab to verify equal loading and efficiency of protein transfer (lower panels). Data is representative of three separate experiments.



**Figure 3.8** Effect of PI3K and MEK inhibitors on IL-1 $\beta$  induced signalling events

$1.2 \times 10^6$  growth arrested, confluent A549 cells were pre-treated with LY294002 (10  $\mu$ M) for 30 mins (A) or with PD98059 (20  $\mu$ M) for 1 hour (B) prior to stimulation at 37°C (for the indicated time points) with IL-1 $\beta$  (10 ng/ml). Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25  $\mu$ g) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted, as described in *Methods and Materials*, with a phospho-specific PKB Ab with affinity for the active Ser<sup>473</sup>-phosphorylated form of PKB or a phospho-specific ERK Ab with an affinity for phosphorylated Thr<sup>202</sup>/Tyr<sup>204</sup> sites. Proteins were visualized with ECL. Blots were stripped and reprobed with anti-PKB or anti-ERK Ab to verify equal loading and efficiency of protein transfer (lower panels). Data is representative of three separate experiments.

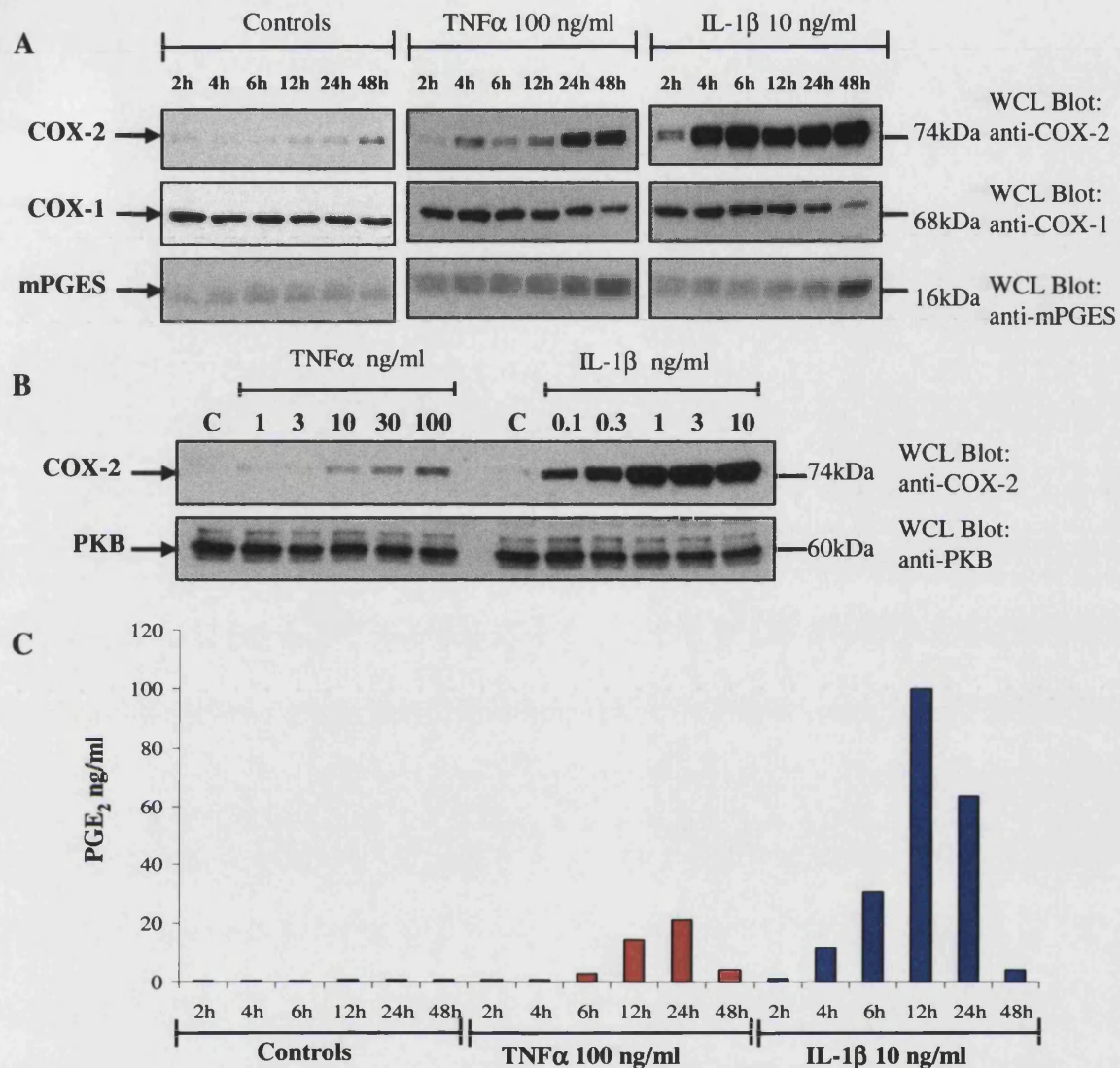
**Induction of COX-2 by TNF $\alpha$  and IL-1 $\beta$** 

It is commonly reported that COX-2 is inducible in other cell lines by stimulation with a variety of pro-inflammatory cytokines, bacterial products, tumour promoters and other mitogens. Therefore the pro-inflammatory Th1 cytokines TNF $\alpha$  and IL-1 $\beta$  were initially chosen in an attempt to induce COX-2 in human lung epithelial cells.

TNF $\alpha$  (100 ng/ml) induced COX-2 protein expression in a bi-phasic manner (Figure 3.9A). The first peak in COX-2 expression was seen at 4 hours, expression then decreased between 6 and 12 hours and a second, more prominent peak in the induction of COX-2 was seen at 24-48 hours post-stimulation. COX-1 expression was unaltered as expected. Production of PGE<sub>2</sub> depends on COX-2 activity generating prostaglandin endoperoxide substrates, which are then subsequently converted to PGE<sub>2</sub> by microsomal PGES (mPGES). An inducible mPGES couples predominantly with COX-2 (Jakobsson et al., 1999; Murakami et al., 2000), whereas a constitutively and widely expressed cytosolic PGES (cPGES) is thought to be coupled to COX-1 (Tanioka et al., 2000). Expression of the inducible mPGES increased in a time dependent manner (Figure 3.9A; middle column lower panel). IL-1 $\beta$  (10 ng/ml) induced COX-2 expression more robustly than TNF $\alpha$  (Figure 3.9A; right column upper panel). This effect also appeared to be bi-phasic in nature, with the first peak seen at 6 hours followed by a second peak in COX-2 expression at 24-48 hours. Furthermore, IL-1 $\beta$  also induced mPGES expression at 48 hours (Figure 3.9A; right column lower panel). Untreated cells showed minimal induction of COX-2 expression at 48 hours, but no effect on mPGES was seen (Figure 3.9A; left

column). On the whole neither cytokine affected COX-1 expression; however a modest effect on COX-1 expression was seen following TNF $\alpha$  and IL-1 $\beta$  stimulation at 48 hours. TNF $\alpha$  and IL-1 $\beta$  induced COX-2 expression was shown to be concentration dependent at 6 hours (Figure 3.9B). The 6 hour time-point was chosen as this was a time-point at which COX-2 induction can be seen clearly but is not maximal, thus ensuring a large window for detection of upregulation or downregulation of the enzyme.

Having established that TNF $\alpha$  and IL-1 $\beta$  induced COX-2 expression, the effects of these cytokines on COX-2 activity was determined by measurement of PGE<sub>2</sub>, a downstream product of COX-2. TNF $\alpha$  and IL-1 $\beta$  induced PGE<sub>2</sub> production in a time-dependent manner which peaked at 12-24 hours post-stimulation (Figure 3.9C). Again, IL-1 $\beta$  showed a more robust induction of PGE<sub>2</sub> than TNF $\alpha$ .



**Figure 3.9 Effect of TNFα and IL-1β on COX-2 and mPGES expression and PGE<sub>2</sub> production.**

$1.2 \times 10^6$  growth arrested confluent A549s were stimulated at 37°C with either TNFα and IL-1β over a time course (2-48 hrs) (A and C). Controls were carried out for each time point.  $1.2 \times 10^6$  growth arrested confluent A549s were stimulated at 37°C for 6 hours with increasing concentrations of TNFα and IL-1β (B). Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25 μg) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted, as described in *Methods and Materials*, with an anti-COX-2 Ab or anti-mPGES Ab (A and B; upper and lower panels respectively). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-COX-1 or anti-PKB Ab to verify equal loading and efficiency of protein transfer (middle panels). Prior to lysing of cells, supernatants were collected and analysed for PGE<sub>2</sub> production by ELISA (C). Samples were assayed in duplicate and results are expressed as mean. Data is representative of three separate experiments.

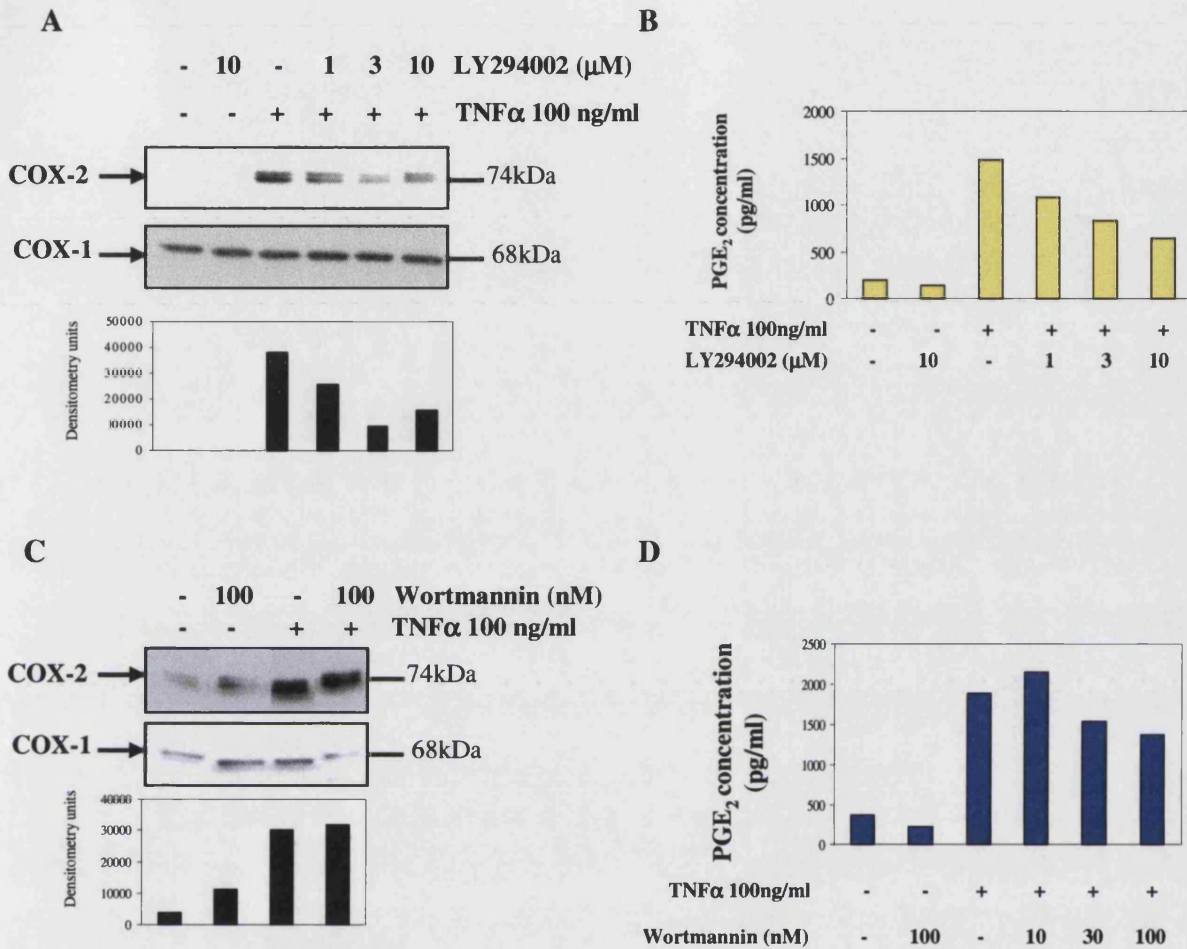
### **Role of PI3K pathway in cytokine induced COX-2 and PGE<sub>2</sub> production**

The role of PI3K pathway in TNF $\alpha$  induced COX-2 expression and PGE<sub>2</sub> production was next investigated by means of two previously characterised PI3K inhibitors, wortmannin and LY294002. COX-2 expression and PGE<sub>2</sub> production were markedly upregulated in response to TNF $\alpha$  (Figure 3.10A and 3.10B) which was sensitive to LY294002 in a concentration dependent manner with a 58% inhibition seen at 10  $\mu$ M. In comparison, TNF $\alpha$  induced COX-2 and PGE<sub>2</sub> production was relatively insensitive to wortmannin (up to 100nM) (Figure 3.10C and 3.10D). Neither inhibitor affected COX-1 expression (Figure 3.10A and 3.10C lower panels).

### **Role of MAPK pathway in cytokine induced COX-2 and PGE<sub>2</sub> production**

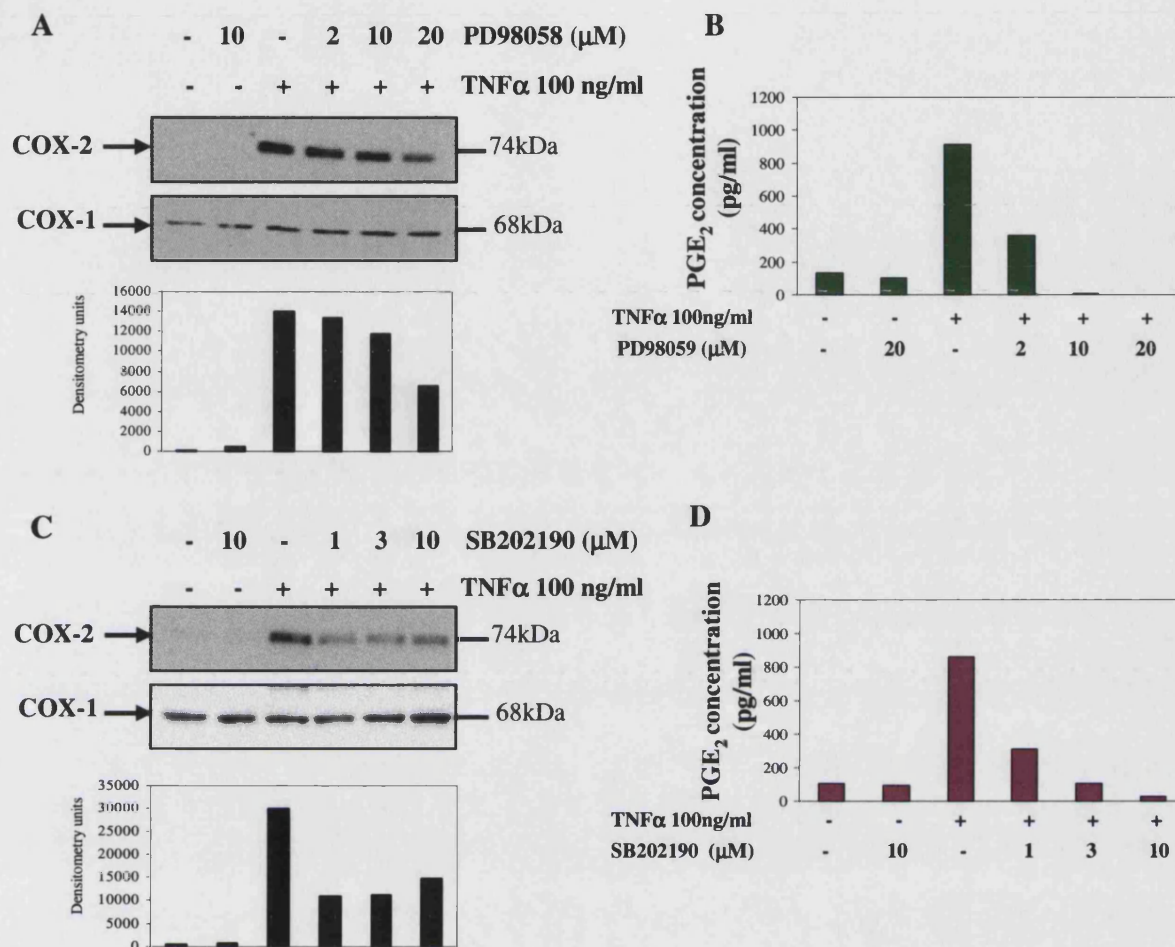
Since TNF $\alpha$  induced activation of the ERK MAPK pathway, the role the ERK MAPK and also the p38 MAPK pathway in TNF $\alpha$  induced COX-2 expression was investigated. This was carried out by use of two MAPK inhibitors, firstly the MEK inhibitor PD98059 and secondly the p38 inhibitor SB202190. PD98059 (2-20  $\mu$ M) partially inhibited TNF $\alpha$  induced COX-2 expression in a concentration dependent manner, with 53 % inhibition seen at 20  $\mu$ M (Figure 3.11A). Furthermore, a more profound effect was seen on PGE<sub>2</sub> production, where complete inhibition of COX-2 activity was demonstrated with PD98059 at concentration of 10-20  $\mu$ M (Figure 3.11B). SB202190 (1-10  $\mu$ M) showed a more marked inhibition of TNF $\alpha$  induced COX-2 expression, with a maximum inhibition of 63 % (Figure 3.11C). Again, a concentration dependent inhibition of PGE<sub>2</sub> production was seen, with a maximal effect being seen at 10  $\mu$ M (Figure 3.11D).





**Figure 3.10 Effect of PI3K inhibitors on TNFα induced COX-2 expression and PGE<sub>2</sub> production**

$1.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 30 mins with LY294002 (1-10 μM) (A) and for 10 mins with another PI3K inhibitor wortmannin (100 nM) (B) prior to stimulation with TNFα 100 ng/ml for 6 hours. Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25 μg) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-COX-2 Ab (upper panels). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-COX-1 Ab to verify equal loading and efficiency of protein transfer (lower panels). Densitometry was carried out on COX-2 blots in order to quantify results and values are graphically represented below the respective blot. Data is representative of three separate experiments.  $1.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 30 mins with LY294002 (1-10 μM) (C) and for 10 mins with another PI3K inhibitor wortmannin (10-100 nM) (D) prior to stimulation with TNFα 100 ng/ml for 24 hours. Supernatants were collected and analysed for PGE<sub>2</sub> production by ELISA. Samples were assayed in duplicate and results are expressed as mean. Data is representative of three separate experiments.



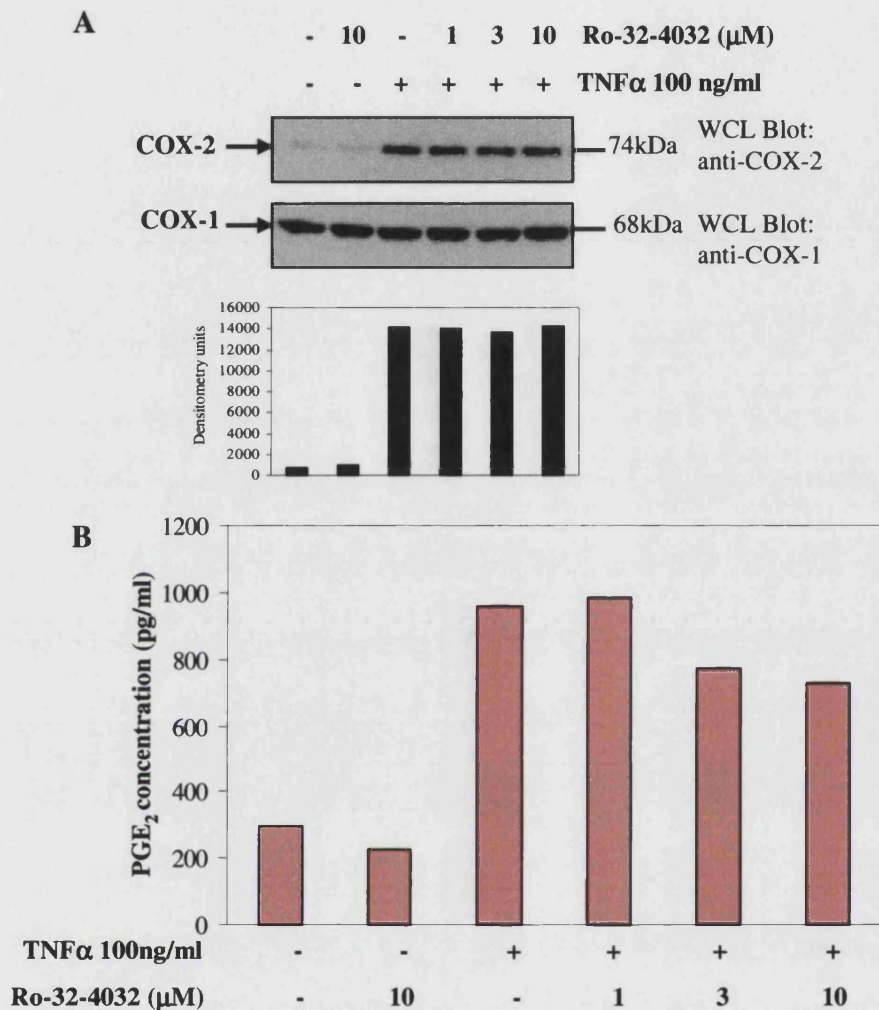
**Figure 3.11 Effect of MEK and p38 inhibitors on TNF $\alpha$  induced COX-2 expression and PGE<sub>2</sub> production**

$1.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 1 hour with PD98059 (2-20  $\mu$ M) (A) and for 1 hour with SB202190 (1-10  $\mu$ M) (B) prior to stimulation with TNF $\alpha$  100 ng/ml for 6 hours. Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25  $\mu$ g) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-COX-2 Ab (upper panels). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-COX-1 Ab to verify equal loading and efficiency of protein transfer (lower panels). Densitometry was carried out on COX-2 blots in order to quantify results and values are graphically represented below the respective blot. Data is representative of three separate experiments.  $1.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 1 hour with PD98059 (2-20  $\mu$ M) (C) and for 1 hour with SB202190 (1-10  $\mu$ M) (D) prior to stimulation with TNF $\alpha$  100 ng/ml for 24 hours. Supernatants were collected and analysed for PGE<sub>2</sub> production by ELISA. Samples were assayed in duplicate and results are expressed as mean. Data is representative of three separate experiments.



### **Role of PKC pathway in cytokine induced COX-2**

Previous studies have indicated the involvement of the PKC pathway in cytokine induced COX-2 expression (Chen et al., 2000). The involvement of this pathway in this system was investigated with the aid of the PKC inhibitor Ro-32-4032. Surprisingly the Ro-32-4032 (1-10  $\mu$ M) had no effect on TNF $\alpha$  induced COX-2 (Figure 3.12A). Ro-32-4032 also had little or no inhibitory effects on TNF $\alpha$  induced PGE<sub>2</sub> production (Figure 3.12B).



**Figure 3.12 Effect of PKC inhibitors on TNFα induced COX-2 expression and PGE<sub>2</sub> production**

$1.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 30 mins with Ro-32-4032 (1-10 μM) prior to stimulation with TNFα 100 ng/ml for 6 hours (A) or 24 hours (B). A- Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25 μg) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-COX-2 Ab (upper panels). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-COX-1 Ab to verify equal loading and efficiency of protein transfer (lower panels). Densitometry was carried out on COX-2 blots in order to quantify results and values are graphically represented below the respective blot. Data is representative of three separate experiments. B- Supernatants were collected and analysed for PGE<sub>2</sub> production by ELISA. Samples were assayed in duplicate and results are expressed as mean. Data is representative of three separate experiments.

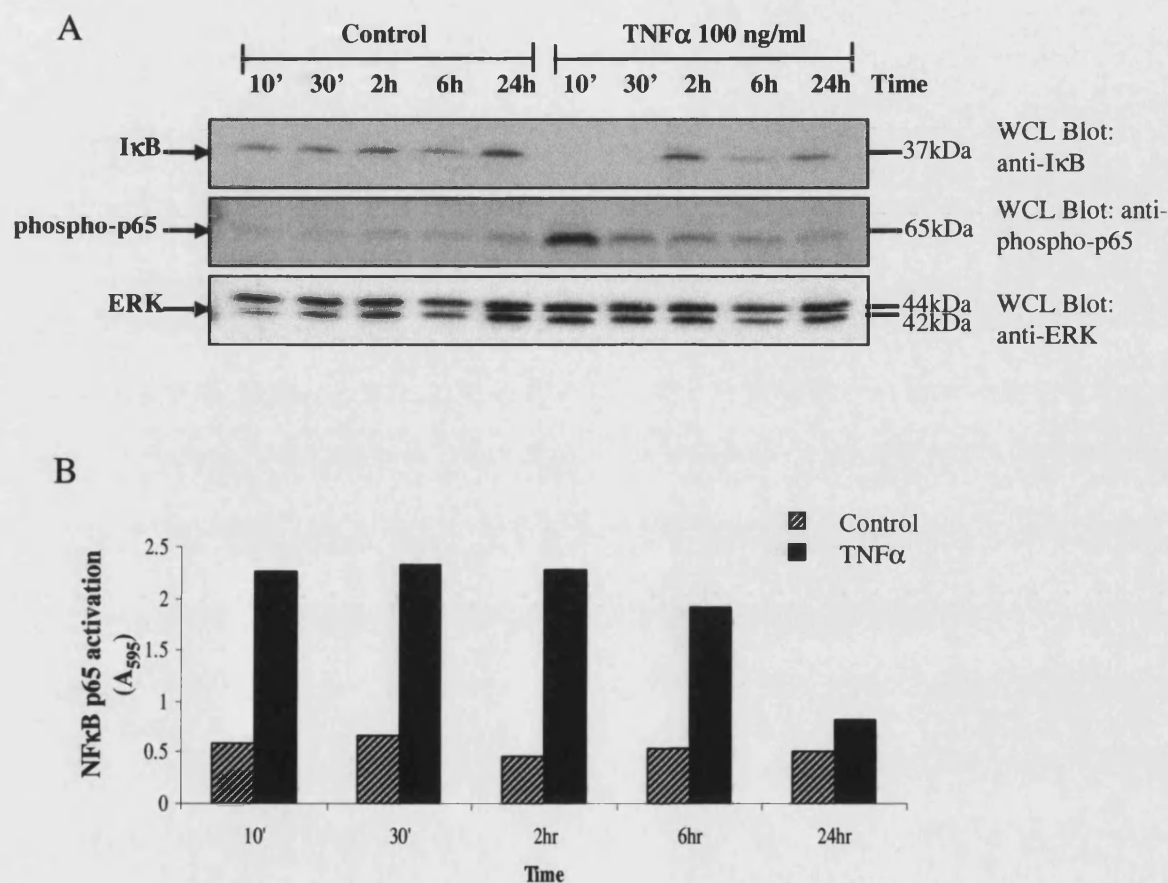
**Involvement of the NFκB pathway in COX-2 regulation by cytokines**

The transcriptional NFκB pathway is known to play an important role in downstream signalling of cytokines (Chen et al., 2000; Hozumi et al., 2001; Haddad, 2002). The COX-2 promoter has NFκB binding sites, and thus NFκB has been shown to play a vital role in the regulation of COX-2 in various cell systems, including A549 cell line (Newton et al., 1997). There are several ways in which the activation of NFκB can be assayed. Firstly, activation of the classical NFκB pathway requires degradation of the inhibitory subunit, IκB. This can easily be assessed by Western analysis using an antibody specific to IκB and determining whether expression of this protein is lost over a time course. Secondly, following IκB degradation the NFκB p65 subunit is phosphorylated and this can also be detected by Western analysis with an antibody specific for NFκB p65 phosphoserine<sup>276</sup>. Thirdly, once NFκB p65 is phosphorylated it is able to translocate to the nucleus and bind specific sites in the promoter region of the target gene, this can be detected by the use of ELISA-based EMSA kits. Finally, NFκB gene reporter assays can be utilised to evaluate the gene activity of NFκB by measuring luciferase activity. This next section employed each of these described assays in order to investigate the role of the NFκB pathway in cytokine induced events.

TNFα (100 ng/ml) induced IκB degradation within 10 mins post-stimulation (Figure 3.13A; upper panel). This effect was maintained until 2 hours, at which point IκB expression was restored. Interestingly a second phase of IκB degradation was seen at 6 hours, and this returned to basal levels by 24 hours. Basal IκB degradation did not alter over the time course of the experiment (Figure 3.13A; upper panel). TNFα

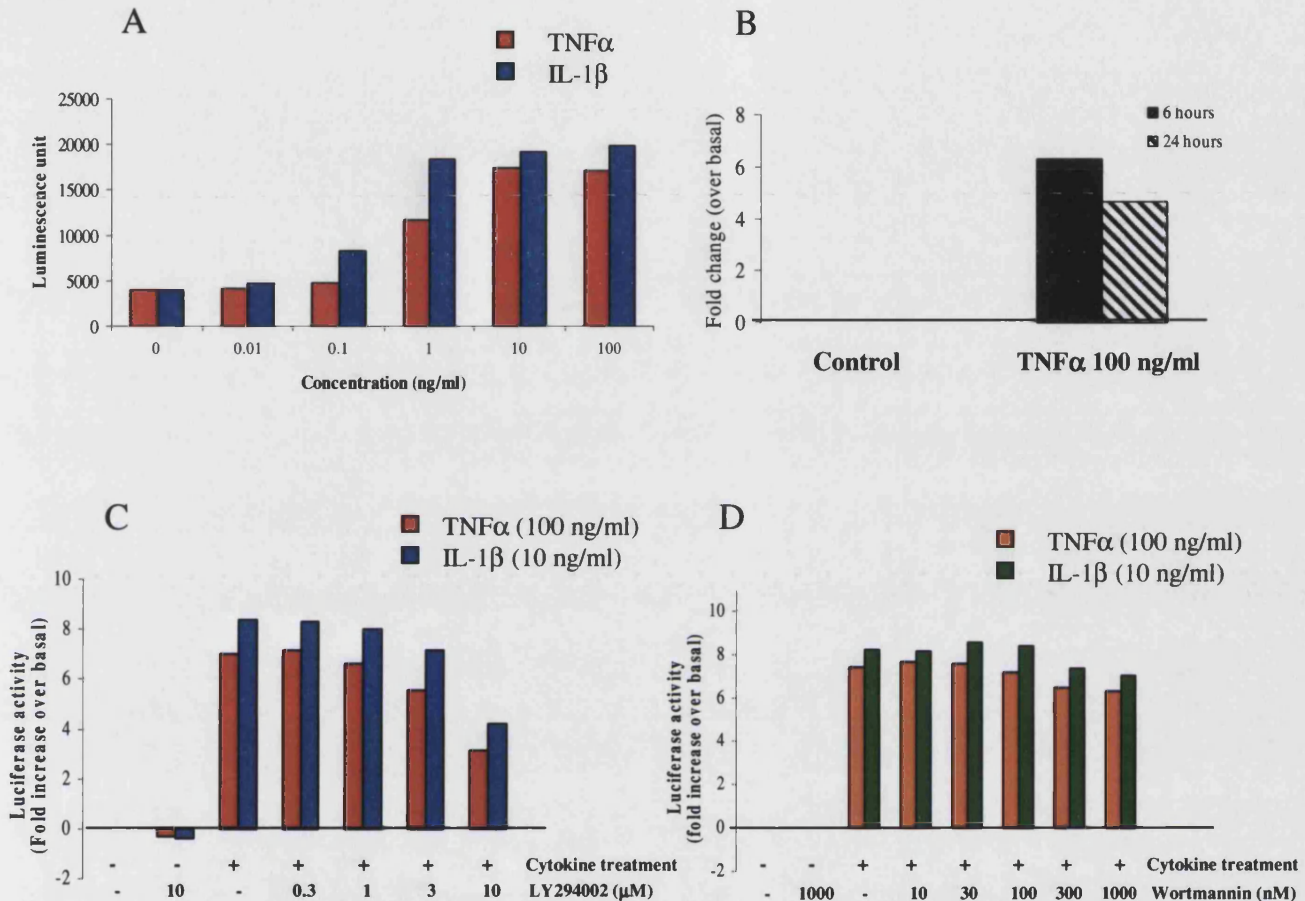
induced marked NF $\kappa$ B p65 phosphorylation at 10 mins post-stimulation, which returned to basal levels by 6 hours (Figure 3.13A; middle panels). The effects seen on NF $\kappa$ B p65 phosphorylation were supported by NF $\kappa$ B p65 activation, which also peaked at 10 mins post-stimulation, was maintained at the same level for 2 hours, after which it declined and almost returned to basal by 24 hours (Figure 3.13B). Time point controls were also carried out and showed no significant changes.

A549 cell line stably transfected with the NF $\kappa$ B luciferase gene reporter construct was utilised to measure reporter activity. TNF $\alpha$  and IL-1 $\beta$  induced NF $\kappa$ B reporter activity in a concentration dependent manner at 6 hour time point (Figure 3.14A). Maximal reporter activity was seen at 10-100 ng/ml TNF $\alpha$  and at 1-100 ng/ml IL-1 $\beta$ . In addition, an increase in reporter activity was still seen at 24 hours post-stimulation with TNF $\alpha$  100 ng/ml albeit slightly lower than that seen at 6 hours (Figure 3.14B). Next it was investigated whether cytokine induced NF $\kappa$ B activity was mediated via PI3K pathway. This was carried out by the use of two PI3K inhibitors previously described. Both TNF $\alpha$  and IL-1 $\beta$  induced NF $\kappa$ B gene reporter activity was sensitive to LY294002 in a concentration dependent manner (Figure 3.14C), with a 50% inhibition seen at 10  $\mu$ M LY294002. In contrast, wortmannin had no effect on TNF $\alpha$  and IL-1 $\beta$  induced NF $\kappa$ B gene reporter activity at concentrations up to 1  $\mu$ M (Figure 3.14D).



**Figure 3.13 Effect of TNFα on IκB degradation, NFκB p65 phosphorylation and activation.**

$3.2 \times 10^6$  growth arrested confluent A549 cells were stimulated with either TNFα 100 ng/ml (A) over a time course (10 mins -24 hrs). Nuclear and cytosolic proteins were extracted as described in *Methods and Materials*. Proteins were quantified and cytosolic protein samples were boiled with 5 X sample buffer, protein (25 μg) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-IκB Ab (upper panels, A) or an anti-phospho-p65 Ab (middle panels, A). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-ERK Ab to verify equal loading and efficiency of protein transfer (lower panels, A). Nuclear extracts (7.5 μg) from the same experiments were assayed for NF-κB p65 activation/DNA binding by use of a TransAM™ NF-κB p65 kit (B). Samples were assayed in duplicate and results are expressed as mean NF-κB p65 activation ( $A_{595}$ ). Data is representative of three separate experiments.



**Figure 3.14** Effect of TNFα and IL-1β on NFκB gene reporter activity.

A-  $0.2 \times 10^6$  growth arrested, confluent A549-κBtkluc cells (A549 cells that have been stably transfected with the NF-κB luciferase gene reporter construct) were stimulated with TNFα or IL-1β (at the indicated concentration) for 6 hours (A); with TNFα (100 ng/ml) at 6 and 24 hours (B); and with TNFα (100 ng/ml) or IL-1β (10 ng/ml) for 6 hours in the presence of 30 min pretreatment with LY294002 (0.3-10 μM; C) or 10 min pretreatment with wortmannin (10-1000 nM; D). Promega Steady-Glo luciferase buffer was added to cells and left at room temperature for 10 mins. Lysates were then mixed and 200 μl sample was then transferred into opaque 96-well plates and luminescence was measured on a Beckman Topcount with a 5 sec luminescence program. Results are expressed as mean luminescence or as fold change over basal. Data is representative of more than three separate experiments.

### **3.3. Summary**

#### **Summary of section 3.1**

- A549 cells express Class IA p85, PI3K-C2 $\alpha$  and PI3K-C2 $\beta$ , but not Class IB p110 $\gamma$ .
- LY294002 and wortmannin show differential selectivity to PI3K isoforms, and similar results were obtained in two distinct cell lines.
- Furthermore, the IC<sub>50</sub> values obtained in the two cell lines are comparable.
- Interestingly wortmannin is unable to inhibit PI3K-C2 $\alpha$  isoforms at concentrations up to 500 nM in either the Jurkat or A549 cell line.
- However, the sensitivity of LY294002 PI3K-C2 $\beta$  can be increased when the amount of substrate (PI) used in the assay is decreased to a minimum of 2.5  $\mu$ g in A549 cell line.

#### **Summary of section 3.2**

- TNF $\alpha$  and IL-1 $\beta$  activated the PI3K and ERK MAPK pathways as assessed by phosphorylation of PKB and ERK respectively.
- TNF $\alpha$  induced phosphorylation of PKB was sensitive to the PI3K inhibitor LY294002 and phosphorylation of ERK was sensitive to the MEK inhibitor PD98059.
- TNF $\alpha$  and IL-1 $\beta$  induced COX-2 expression in a concentration dependent manner. In addition, both cytokines induced the time dependent production of PGE<sub>2</sub>, a downstream product of COX-2.

- TNF $\alpha$  induced COX-2 expression and PGE<sub>2</sub> production was sensitive to the PI3K inhibitor LY294002, but insensitive to another PI3K inhibitor wortmannin.
- TNF $\alpha$  induced COX-2 expression and PGE<sub>2</sub> was also sensitive to the MEK inhibitor PD98059 and the p38 inhibitor SB202190, but was insensitive to the PKC inhibitor Ro-32-4032.
- TNF $\alpha$  activated the NF $\kappa$ B pathway, as shown by time dependent I $\kappa$ B degradation, NF $\kappa$ B p65 phosphorylation and activation, and concentration dependent induction of NF $\kappa$ B gene reporter activity. Furthermore, IL-1 $\beta$  induced NF $\kappa$ B gene reporter activity in a concentration dependent manner. Both TNF $\alpha$  and IL-1 $\beta$  induced NF $\kappa$ B gene reporter activity was sensitive to PI3K inhibitor LY294002, but insensitive to wortmannin.



### **3.4. Discussion I**

#### **Characterisation of PI3K isoform expression and inhibitor sensitivity**

This work looks at the involvement of signalling pathways in the regulation of COX-2 expression at the protein and product level by TNF $\alpha$  and IL-1 $\beta$ . Previous reports have only shown the expression of p110 $\alpha$  in A549 cell line (Ahmad et al., 2003). This study reports for the first time expression of Class IA PI3K, PI3K-C2 $\alpha$  and PI3K-C2 $\beta$  in A549 cell line. It was not surprising that the A549 cell line did not express p110 $\gamma$ , as this isoform is known to be restricted to haematopoietic lineages (Vanhaesebroeck and Waterfield, 1999). The expression of PI3K isoforms has been shown to be expressed in many cells associated with lung inflammation. Furthermore, studies have shown that PI3K activity increased significantly after allergen challenge in a murine model of asthma (Kwak et al., 2003). Administration of either wortmannin or LY294002 reduced bronchial inflammation and airway hyperresponsiveness. Thus these studies suggest an important role of PI3K in airway inflammation.

The study of the role of PI3Ks in cell physiology has been aided by the use of the inhibitors wortmannin and LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one]. Wortmannin is a PI3K inhibitor derived from a metabolite of the fungus *Penicillium funiculosum* Thom (Wiesinger et al., 1974; Park et al., 1997). It was first characterized as an inhibitor that is effective at micromolar concentrations of myosin light-chain kinase but was later shown to inhibit mammalian PI3Ks at nanomolar concentrations (Arcaro and Wymann, 1993; Powis et al., 1994). It

covalently attaches to the p110 catalytic subunit and causes irreversible inhibition with a  $K_i$  of 1-10 nM and a similar  $IC_{50}$ . LY294002 is a structurally distinct, competitive inhibitor for the adenosine triphosphate binding site of PI3K and has been shown to cause specific inhibition with a  $K_i$  of 1.6  $\mu$ M and an  $IC_{50}$  of 1.4  $\mu$ M (Vlahos et al., 1994).

Domin et al. (1997), demonstrated that wortmannin is a poor inhibitor of PI3K-C2 $\alpha$ . In this study, it is demonstrated here that PI3K-C2 $\alpha$  was refractory to wortmannin in both Jurkat and A549 cell line. LY294002 was shown to be less selective towards PI3K-C2 $\beta$  compared to the other isoforms investigated and this was in accordance with studies carried out by Arcaro et al., (1998). The differential sensitivity of PI3K isoforms to LY294002 and wortmannin must therefore be taken into account when utilising these inhibitors to evaluate the role of PI3K in various systems.

Another possible explanation for the differential effects of the two inhibitors is that wortmannin and LY294002 may not be as specific at inhibiting PI3K as claimed. Although there are reports demonstrating the specificity of their actions (Wiesinger et al., 1974; Vlahos et al., 1994; Davies et al., 2000), there are isolated reports suggesting other actions of these pharmacological compounds. Wortmannin has been reported to inhibit phospholipase A<sub>2</sub> (Cross et al., 1995), although if this resulted in less arachidonic acid as substrate for COX-2 one might expect inhibition of PGE<sub>2</sub> production rather than potentiation. Reports that wortmannin can inhibit PI4K (Nakanishi et al., 1995) are more difficult to interpret, as is the finding that LY294002 can inhibit casein kinase (Davies et al., 2000).

Although disparity between results obtained using wortmannin and LY294002 are rare, there is a precedent (Salh et al., 1998). This group were studying PI3K and iNOS activation in a macrophage cell line and showed that activation of PI3K by LPS was sensitive to both wortmannin and LY294002. However, nitric oxide generation was sensitive only to LY294002 and not to wortmannin. They proposed a FKBP12-rapamycin-associated protein (FRAP) dependent pathway which was more sensitive to LY294002 than to wortmannin. Supporting their proposition is evidence from a second group demonstrating that both wortmannin and LY294002 can inhibit FRAP at higher concentrations than those necessary to inhibit PI3K (Brunn et al., 1996).

Interestingly, when the amount of substrate used in the assay was reduced, PI3K-C2 $\beta$  activity was more sensitive to LY294002. Since LY294002 is known to act by competitively inhibiting the ATP binding site, it can be hypothesised that high concentrations of substrate (PI) may somehow be hindering the ability of LY294002 to bind to this site. Thus these points must be taken into consideration when interpreting these results since it is difficult to determine the substrate concentration in these cell systems.

To summarise, one must be aware of the potential pitfalls regarding the interpretation of the results when using these pharmacological inhibitors of PI3K. There are other methods of inhibiting PI3K such as molecular methods like micro-injection of antibodies or transferring dominant negative forms of PI3K into cells (Benistant et al., 2000; Sasaki et al., 2000; Stein and Waterfield, 2000; Weaver and Ward, 2001). Similar transfer methods can be used to manipulate PI3K by causing

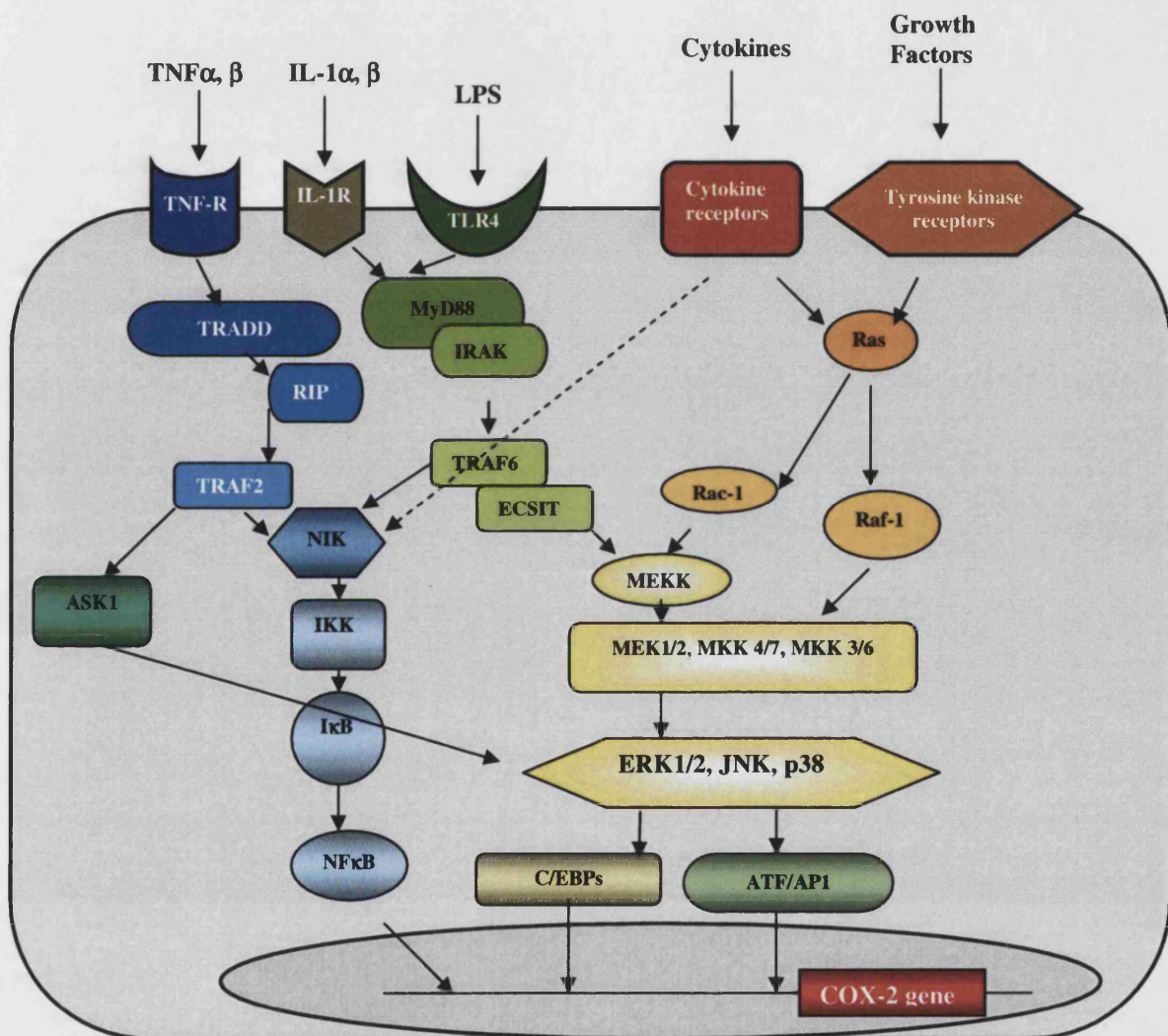
its over-expression, and these may be placed under the control of an environmental agent such as an antibiotic in the culture media (Craddock et al., 1999). Evidence should be gathered from different models using different mechanisms of manipulating PI3K and from the resulting evidence a consensus should be drawn. Thus more selective assays as well as inhibitors and/or molecular methods would be required to fully investigate the role of individual PI3K isoforms in the regulation of COX-2 expression in lung epithelial cells.

### **TNF $\alpha$ and IL-1 $\beta$ mediated signalling**

The role of PI3K and MAPK in TNF $\alpha$  and IL-1 $\beta$  signalling has been established (Saklatvala et al., 1999; Akira, 2003). The activation of PI3K occurs through an interaction of the SH2 domain of the PI3K p85 subunit with a domain in the partner cell surface receptor containing the motif YXXM (Saklatvala et al., 1999; Akira, 2003). A putative PI3K binding site (amino acids 257–260, YKAM) is found in the C terminus of MyD88 (molecule myeloid differentiation factor 88), an adaptor which links IL-1R with downstream signaling molecules. Activation of the toll-like receptor (TLR)4 with LPS or the IL-1 receptor with IL-1 $\beta$  induces the recruitment of MyD88. MyD88 in turn recruits two distinct putative serine-threonine kinases. One kinase is the IL-1 receptor-activated kinase (IRAK), IRAK then interacts with the adaptor molecule, TNF receptor-activated kinase (TRAF6), which links both proteins to a second protein kinase NF $\kappa$ B-inducing kinase (NIK). NIK activates the IKK that directly phosphorylates I $\kappa$ B. The phosphorylation of I $\kappa$ B initiates its ubiquitin-proteasome-mediated degradation, and liberates and activates NF $\kappa$ B. Moreover, the TNF-receptor activates TRAF2, but not TRAF6. Downstream

signalling pathways of LPS, IL-1 and TNF converge at a point distal to TRAF6 and TRAF2 (Figure 3.15).

The involvement of the MAPK and NF $\kappa$ B pathway in TNF $\alpha$  and IL-1 $\beta$  signalling has been well established.



**Figure 3.15. Signalling pathways activated downstream of surface receptors leading to gene activation** (adapted from (Tanabe and Tohnai, 2002)).

Here it is shown that both the PI3K and ERK MAPK pathways are activated following TNF $\alpha$  and IL-1 $\beta$  stimulation in lung epithelial cell line A549. As expected

the PI3K inhibitor LY294002 completely abolished TNF $\alpha$  and IL-1 $\beta$  induced PKB phosphorylation. In contrast the MEK inhibitor, PD98059, although it showed attenuation in the TNF $\alpha$  and IL-1 $\beta$  induced ERK phosphorylation did not completely abolish the response.

### **Activation of the COX-2 pathway by TNF $\alpha$ and IL-1 $\beta$**

Several studies have shown that cytokines such as TNF $\alpha$  and IL-1 $\beta$  can induce COX-2 expression in lung epithelial cell lines (Newman et al., 1994; Newton et al., 1997; Chen et al., 2000; Lin et al., 2000; Chen et al., 2001). Of particular importance are reports that show the involvement of various signalling pathways such as NF $\kappa$ B, PKC and MAPK in the induction of COX-2 by both TNF $\alpha$  and IL-1 $\beta$  (Newton et al., 1997; Chen et al., 2000; Lin et al., 2000; Chen et al., 2001). The PI3K pathway has also been shown to play a regulatory role in TNF $\alpha$  induced COX-2 expression in colonic epithelial cell lines (Weaver et al., 2001).

There are many signalling pathways involved in COX-2 gene induction some of which have already been outline in the above section. c-Jun also appears to play a role in COX-2 activation in LPS-treated macrophages. Interestingly, MEKK1 and JNK-dependent transcriptional activation of the COX-2 gene in LPS-treated macrophages does not require Ras activation. Recently, the adapter protein ECSIT, has been identified and shown to link the signalling pathway from the cytoplasmic domain of receptors (TLR4 and IL-1 receptor) to MEKK1 (Figure 3.15).

The A549 cell line used in this study displayed a small amount of constitutive COX-2 expression at the protein level which is faintly seen in blots with good sensitivity. This is in keeping with results from other groups (Pawliczak et al., 2004; Petkova et al., 2004). The small amount of constitutive expression seen is reflected in detectable amounts of PGE<sub>2</sub> production.

It has been demonstrated here that the pro-inflammatory cytokines TNF $\alpha$  and IL-1 $\beta$  can induce COX-2 in the A549 cell line. TNF $\alpha$  (and to a lesser extent IL-1 $\beta$ ) induce COX-2 expression in a bi-phasic manner. This has also been shown in the carrageenin-induced pleurisy model in rats (Gilroy et al., 1999), where COX-2 protein expression peaked initially at 2 hrs, associated with maximal PGE<sub>2</sub> synthesis. However at 48 hrs there was a second increase in COX-2 expression. Paradoxically, this coincided with inflammatory resolution and was associated with minimal PGE<sub>2</sub> synthesis. In contrast, levels of PGD<sub>2</sub> and 15d-PGJ<sub>2</sub> were high at 2 hrs, decreased as inflammation increased, but were increased again at 48 hrs. The use of a selective COX-2 inhibitor and a dual COX-1/COX-2 inhibitor blocked inflammation at 2 hours but significantly exacerbated inflammation at 48 hrs, which was associated with reduced exudate PGD<sub>2</sub> and 15d-PGJ<sub>2</sub> concentrations, and was also reversed by replacement of these PGs. Thus this study suggested that COX-2 may be pro-inflammatory during the early phase of a carrageenin-induced pleurisy, but may aid resolution at the later phase by generating an alternative set of anti-inflammatory PGs (Gilroy et al., 1999). In the A549 cell system PGE<sub>2</sub> concentration peaked between 12-24 hrs following stimulation with either TNF $\alpha$  or IL-1 $\beta$ . The profile of PGE<sub>2</sub> production did not follow the same kinetics of COX-2 expression, in that at late time points (48 hrs) the level of PGE<sub>2</sub> produced was low despite maximal COX-

2 expression. This effect may suggest that the profile of PG produced by COX-2 is altered at later time points, reflecting the resolution phase of inflammation as described by work done by Gilroy et al., (1999).

COX-1 expression was unaltered following cytokine treatment, consistent with its role as the “housekeeping” isoform. mPGES is coupled to COX-2 and thus the increase in the mPGES expression was not unexpected since COX-2 expression increased following cytokine stimulation. The putative promoter of the human mPGES-1 gene is GC-rich, lacks a TATA box, and contains binding sites for C/EBP  $\alpha$  and  $\beta$  and AP-1, two tandem GC- boxes, two progesterone receptor and three GRE elements (Naraba et al., 2002). Of these sites, the GC boxes are critical for the promoter activity, where the transcription factor Egr-1 binds to the proximal GC box and triggers mPGES-1 transcription. Stimulus-induced activation of MAPK leads to the induced expression of the transcription factor Egr-1 (Guha et al., 2001). Furthermore, Egr-1 is phosphorylated and activated by MAPK. In the context of my findings, both TNF $\alpha$  and IL-1 $\beta$  were able to activate MAPK which may induce activation of Egr-1. Activated Egr-1 can bind to the proximal GC box in the mPGES-1 promoter, leading to transcriptional activation of the mPGES-1 gene (Murakami and Kudo, 2004). Although the role of Egr-1 in mPGES induction has been documented, the role of NF $\kappa$ B is also thought to be of importance (Catley et al., 2003). This is of particular interest in this system since results show activation of the classical NF $\kappa$ B pathway.



### **Involvement of signalling pathways in TNF $\alpha$ induced COX-2 expression**

As previously outlined signalling downstream of TNF and IL-1 receptors can activate the PI3K pathway. The PI3K pathway lies upstream of TNF $\alpha$  induced NF $\kappa$ B signalling (Ozes et al., 1999; Reddy et al., 2000), and since the COX-2 promoter contains NF $\kappa$ B binding sites it can be hypothesised that these pathways may play important roles in cytokine induced gene expression.

#### *PI3K pathway*

The involvement of the PI3K pathway in TNF $\alpha$  induced COX-2 expression and PGE<sub>2</sub> production is unclear given that the two PI3K inhibitors utilised showed differential effects. LY294002 showed inhibitory effects on TNF $\alpha$  induced COX-2 expression and PGE<sub>2</sub>, whereas wortmannin showed no significant effect on either TNF $\alpha$  induced COX-2 expression or PGE<sub>2</sub> production. Differential effects of PI3K inhibitors on COX-2 expression have previously been demonstrated (Weaver et al., 2001). However, Weaver et al., showed that wortmannin further enhanced cytokine-induced COX-2 expression in colonic epithelial cells, and in comparison LY294002 enhanced cytokine-induced COX-2 at low concentrations but had an inhibitory effect at higher concentration (10  $\mu$ M) (Weaver et al., 2001). The inhibitory effects of LY294002 at higher concentrations in the study by Weaver et al., were suggested to be due to non-specific effects or due to disparate effects on different PI3K isoforms. The disparate effects seen in my system may be due to the differential sensitive of the two inhibitors on activity of different PI3K isoforms. Since PI3K-C2 $\alpha$  is refractory to wortmannin and has no effect on TNF $\alpha$  induced NF $\kappa$ B reporter activity and COX-2, this isoform may be of particular importance in TNF $\alpha$  induced events.

### *MAPK pathway*

The data presented here also suggests an important role of the MAPK pathway in TNF $\alpha$  induced COX-2 expression. Both the MEK inhibitor, PD98059 and the p38 MAPK inhibitor partially abrogated TNF $\alpha$  induced COX-2 expression. Furthermore, TNF $\alpha$  induced phosphorylation of ERK MAPK in A549. These pathways have previously been shown to play regulatory roles in COX-2 expression (Yang et al., 2002). Inflammatory stimuli activate major intracellular signalling pathways such as the: NF $\kappa$ B pathway and the MAPK pathways (Ono and Han, 2000; Kracht and Saklatvala, 2002; Clark et al., 2003; Kumar et al., 2003). The MAPK are inactivated by phosphatases, especially the dual specificity phosphatases. These pathways drive transcription of inflammatory genes. The promoter regions of these genes, such as COX-2, have binding sites for a limited set of transcription factors: sites for NF $\kappa$ B, AP-1, C/EBP $\beta$  and Ets family members are common. JNKs phosphorylate and activate c-jun and ATF2, which are components of AP-1 binding complexes. ERK activates C/EBP $\beta$  and AP-1, and all three MAPKs can phosphorylate Ets transcription factors such as Elk-1 and SAP-1 (Kracht and Saklatvala, 2002).

Several transcription factors can be phosphorylated by p38 and the downstream kinases might also be involved (Ono and Han, 2000; Kracht and Saklatvala, 2002) in the induction of COX-2. The p38 pathway stabilises inflammatory response protein mRNAs (Kracht and Saklatvala, 2002; Clark et al., 2003) and promotes their translation (Kumar et al., 2003) through AU-rich elements (AREs) in the 3' untranslated region of the mRNAs (Winzen et al., 1999; Lasa et al., 2000). These contain repeats of the motif AUUUA, often in a U-rich context, and are present in

mRNAs of cytokines, inflammatory proteins and oncogenes (Bakheet et al., 2001; Kracht and Saklatvala, 2002; Clark et al., 2003). They were discovered as instability elements but, in the case of inflammatory response mRNAs, the instability is countered by signalling in the p38 pathway (Winzen et al., 1999; Lasa et al., 2000; Frevel et al., 2003). COX-2 mRNA has an ARE in the proximal 3' untranslated region, which has been mapped in detail (Lasa et al., 2000; Sully et al., 2004); it is stabilised by MAPKAPK-2. The ARE directs rapid de-adenylation of the 3'-polyA tail of the mRNA; this is followed by rapid degradation of the body by the exosome, a complex of 3'→ 5' nucleases. Activation of p38 markedly delays the de-adenylation but has little effect on the rate of decay of the de-adenylated message (Dean et al., 2003). Thus it is clear that p38 plays an important role in the regulation of COX-2 mRNA stability, and thus is vital in the expression of COX-2.

#### *PKC pathway*

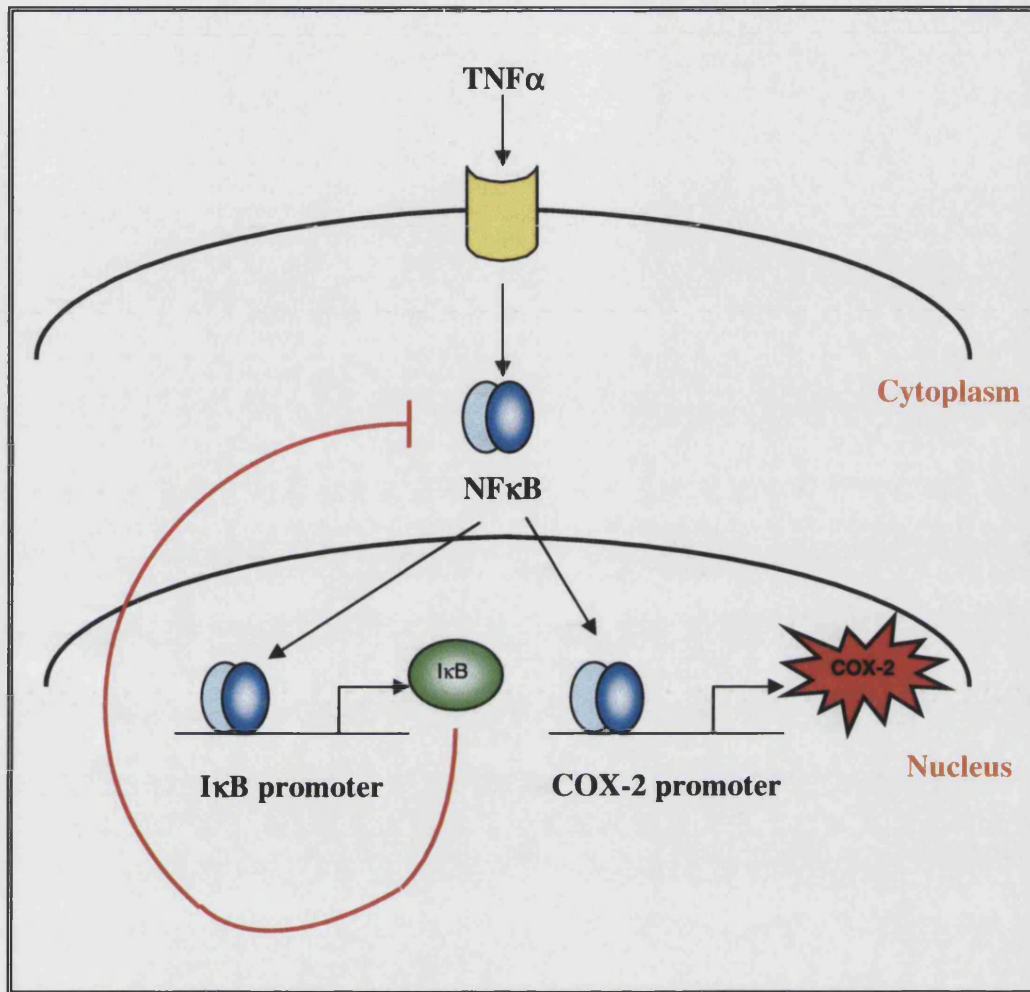
Despite evidence that suggested the involvement of the PKC pathway in COX-2 regulation in lung epithelial cell line, NCI-H292 (Chen et al., 2000), this pathway was not an obligatory event in TNF $\alpha$ -mediated COX-2 expression in A549 cells.

#### **TNF $\alpha$ induced activation of the NF $\kappa$ B pathway**

As outlined in the introduction the NF $\kappa$ B pathway plays a fundamental role in the regulation of many inflammatory mediators including COX-2. Previous studies in the A549 cell line have shown the importance of the NF $\kappa$ B pathway in COX-2 and mPGES expression and PGE<sub>2</sub> production following IL-1 $\beta$  stimulation (Catley et al.,

2003). However, this effect has not been conclusively shown to involve the PI3K pathway in this system. The data presented here clearly indicated activation of the classical NF $\kappa$ B pathway following cytokine stimulation. TNF $\alpha$  and IL-1 $\beta$  induced NF $\kappa$ B gene reporter activity, was sensitive to the PI3K inhibitor LY294002 but insensitive to another PI3K inhibitor wortmannin. Again this result can be explained due to the differential selectivity of LY294002 and wortmannin on PI3K isoforms. The above results suggest that cytokine mediated activation of the NF $\kappa$ B pathway is partially dependent on the PI3K pathway. Contrary to the results illustrated here, the PI3K pathway has been shown to negatively regulate the NF $\kappa$ B pathway in other systems (Weaver et al., 2001).

The bi-phasic induction of COX-2 protein by TNF $\alpha$  and IL-1 $\beta$  was discussed earlier. Interestingly TNF $\alpha$  also induced I $\kappa$ B degradation in a bi-phasic manner. These results may therefore suggest that the bi-phasic nature of I $\kappa$ B degradation leads to bi-phasic induction of COX-2 since the COX-2 promoter is known to have NF $\kappa$ B binding sites. Furthermore, the I $\kappa$ B promoter also has NF $\kappa$ B binding sites and thus activation of this pathway has previously been shown to lead to autoregulation (Cogswell et al., 1993; Ito et al., 1994; Hoffmann et al., 2002). Figure 3.16 outlines the suggested autoregulatory mechanism of the NF $\kappa$ B pathway.

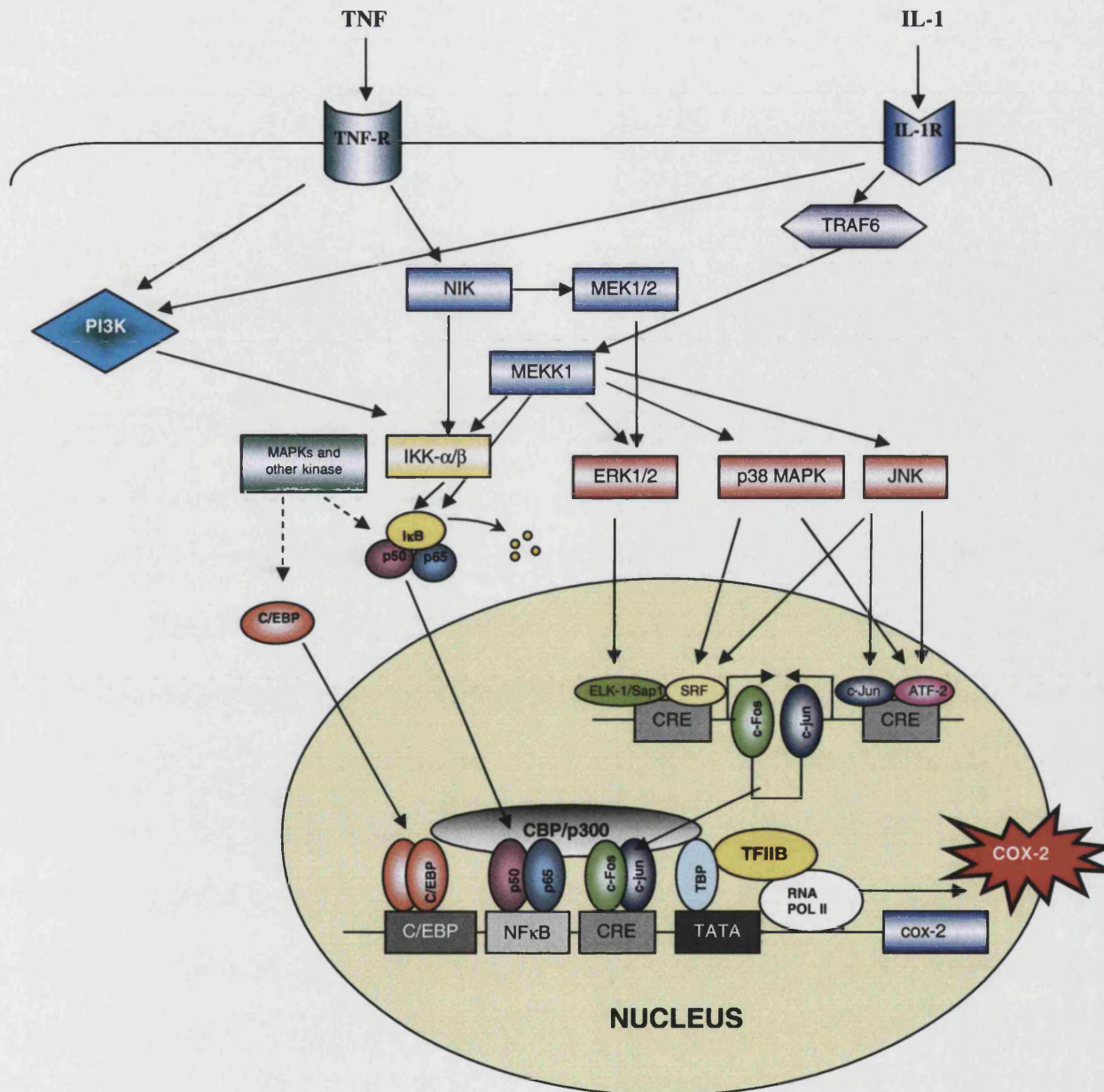


**Figure 3.16. Autoregulation of the NFκB pathway by IκB.**

Several studies have shown TNFα stimulation does not induce p100 processing, although it strongly induces IκBα degradation (Coope et al., 2002; Derudder et al., 2003; Gommerman and Browning, 2003; Muller and Siebenlist, 2003). However, TNFα stimulation does increase the expression of both p100 and RelB, which are transcriptional targets of the classical NFκB signalling pathway (Gommerman and Browning, 2003; Yilmaz et al., 2003). Thus the classical NFκB pathway is indirectly

linked to the alternative NF $\kappa$ B pathway and may influence the amplitude and duration of its activation.

Mutation analysis of p65 Ser276 has shown the extreme importance of this residue for engagement of the cofactor CBP and its paralogue p300 (Zhong et al., 1998). MSK1 is a direct target for both p38 and ERK MAP kinases and was first identified as a very potent CREB kinase (Deak et al., 1998). Alignment of the CREB sequence containing the MSK1 phosphorylation site (Ser133) and the sequence surrounding Ser276 of p65 revealed an unexpectedly high degree of similarity. The fact that phosphorylation of CREB and/or p65 is essential for recruitment of CBP/p300 led Vermeulen et al., to investigate the role of MSK1 in p65 phosphorylation. Indeed the authors showed that MSK1 is involved in Ser276 phosphorylation and subsequent gene activation in response to TNF treatment (Vermeulen et al., 2003)



**Figure 3.17. Intracellular signalling pathways thought to mediate COX-2 induction in A549 cells.** A distinct set of upstream kinases including MAPKs can activate transcription factors (NFκB, AP-1, C/EBP) which in turn leads to upregulation of COX-2 (adapted from Chun et al., 2004). Putative pathways are shown in solid arrows whereas unknown pathways are shown by dashed arrows.

Although this chapter has shown that TNFα and IL-1β activate the NFκB pathway, the involvement of other transcriptional pathways in the induction of COX-2 cannot

be ruled out. Figure 3.17 outlines the intracellular signalling pathway that I found to be involved in mediating COX-2 induction.

Due to the clear evidence of the role of the MAPK pathway in the induction of COX-2 expression it is highly unlikely that NFκB is the sole transcriptional pathway involved in the cytokine induced expression of COX-2 in A549 cell line.

### **3.5. Conclusions**

This chapter has outlined some of the signalling pathways involved in the regulation of COX-2 expression by cytokines in A549, lung epithelial cell line. The MAPK, NFκB and to a certain extent the PI3K pathway all seem to play important roles in this process. However in this chapter I have also characterised two PI3K inhibitors and highlighted the potential pitfalls of the use of these inhibitors as pharmacological tools.



# **4.**

## **RESULTS II**

## **4. RESULTS II**

### **4.1 Regulation of signalling and basal COX-2 expression and activity by PPAR $\gamma$ ligands**

#### **4.1.1. Background**

PPAR $\gamma$  expression is augmented in the bronchial submucosa, airway epithelium and smooth muscle of steroid-untreated asthmatics, as compared with control subjects (Benayoun et al., 2001), suggesting a role of PPAR $\gamma$  in airway inflammation.

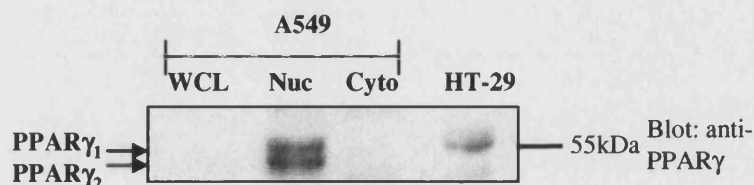
PPAR $\gamma$  ligands have been shown to inhibit production of many pro-inflammatory mediators as outlined in the introduction. Several studies have investigated the role of PPAR $\gamma$  ligands in modifying animal models of autoimmune diseases and further support the hypothesis that PPAR $\gamma$  ligands may be beneficial in inflammatory responses (Nakajima et al., 2001; Cuzzocrea et al., 2003).

This chapter investigates the effects of PPAR $\gamma$  ligands on signalling pathways and COX-2 expression, and the effect of PPAR $\gamma$  ligands on cytokine induced inflammatory responses.

#### **4.1.2. Results**

##### **Expression of PPAR $\gamma$ in A549 cell line**

PPAR $\gamma$  has shown to be expressed in various epithelial cells including colonic epithelial cells, mammary epithelial cells and also lung epithelial cells (Benayoun et al., 2001; Ikawa et al., 2001; Subbaramaiah et al., 2001). In order to evaluate the role of PPAR $\gamma$  on the regulation of COX-2 by PPAR $\gamma$  ligands, its expression was first determined by immunoblotting. Some studies indicate that PPAR $\gamma$ , like other nuclear receptors, resides in the cytoplasm until activated, and then translocates to the nucleus where it can regulate transcription. In contrast, immunofluorescence analysis and western blot analysis have shown PPAR $\gamma$  to be predominately nuclear localised in macrophages (Chinetti et al., 1998) and nuclear localisation of PPAR $\gamma$  has been shown to be independent of ligand (Dreyer et al., 1993). Thus the localisation of PPAR $\gamma$  in its inactive state may vary from one cell type to another. It was therefore necessary to not only verify the expression of PPAR $\gamma$  in A549 cell line, but also to determine its localisation within the cell. A colonic epithelial cell line (HT-29) was used as a positive control, since this cell line has been shown to express PPAR $\gamma$  (DuBois et al., 1998; Lefebvre et al., 1999; Galetto et al., 2001; Ikawa et al., 2001; Kehrer et al., 2001). Nuclear and cytoplasmic extracts were also compared to whole cell lysates obtained with standard lysis buffer. The molecular weight of PPAR $\gamma$  is around 54 kDa. Figure 4.1 shows the expression of PPAR $\gamma_1$  and PPAR $\gamma_2$  in the nucleus of A549 and HT-29 cell lines. In addition the HT-29 cell line shows expression of PPAR $\gamma$  in whole cell extract.



**Figure 4.1** The A549 cell line express PPAR $\gamma$  isoforms.

$3.2 \times 10^6$  grow-arrested, confluent A549 cells were harvested for nuclear and cytosolic protein extraction as described in *Methods and Materials*. Positive control lysates (nuclear extract of HT-29 colonic epithelial cell line for PPAR $\gamma$  expression) were also collected. Proteins were quantified by Bradford assay and boiled with  $5 \times$  sample buffer. Protein ( $25\mu\text{g}$ ) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted, as described in *Methods and Materials*, with an anti-PPAR $\gamma$  Ab (which recognises PPAR $\gamma_1$  and PPAR $\gamma_2$ ). Proteins were visualized with ECL. Data is representative of three experiments.

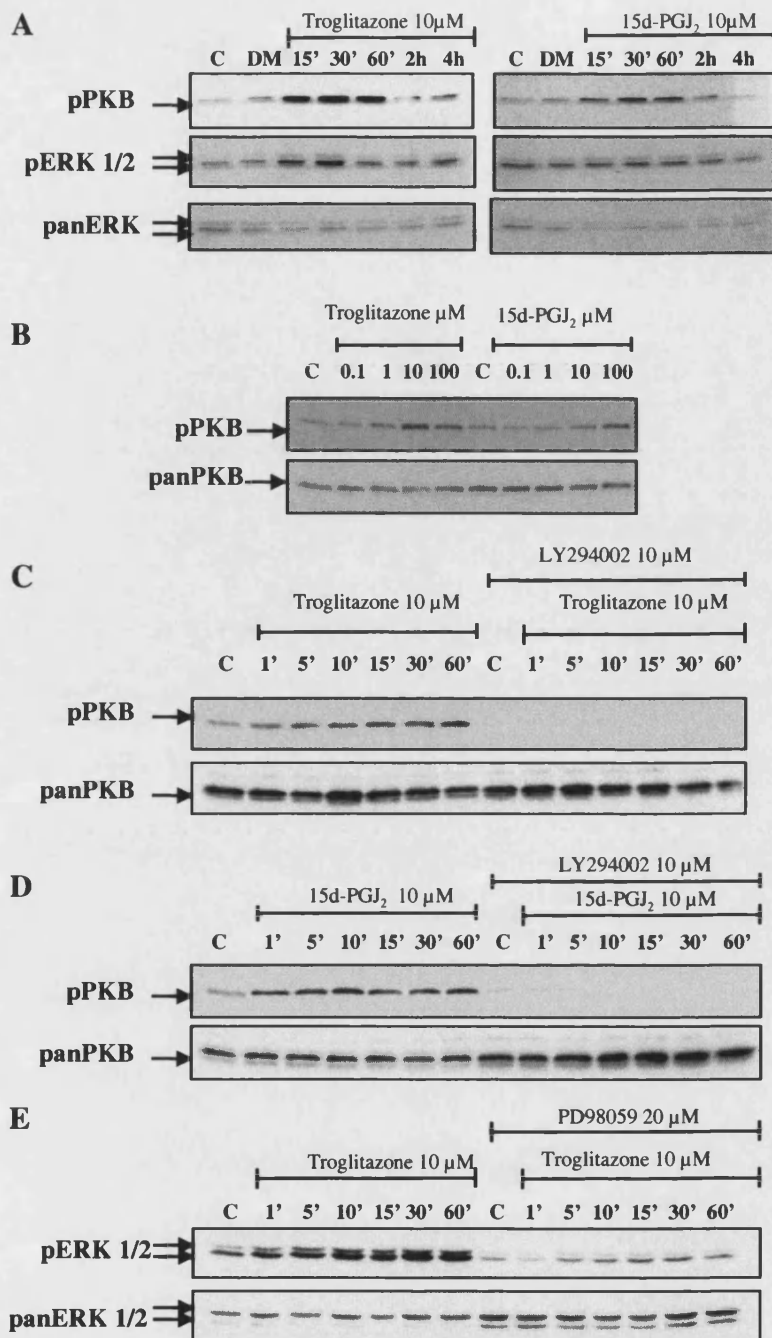
### **Effect of PPAR $\gamma$ ligands on PKB and ERK phosphorylation**

PPAR $\gamma$  ligands have been shown to activate both the PI3K and extracellular regulated kinase ERK MAPK pathways in vascular smooth muscle cells (Takeda et al., 2001) and the ERK MAPK pathway in murine myoblasts (Huang et al., 2002).

Having established that A549 cells express PPAR $\gamma$ , and PI3K isoforms (in Chapter 3), the involvement of the PI3K pathway in PPAR $\gamma$  ligand signalling in A549 cells was investigated. PPAR $\gamma$  and PI3K pathways have been shown to be involved in the regulation of COX-2 expression in other cell lines following stimulation with a variety of stimuli (Matsuura et al., 1999; Chen et al., 2000; Weaver et al., 2001; Yang et al., 2002; Liu et al., 2003; Luo et al., 2003; Bradbury et al., 2004; Chen et al., 2004; Lin et al., 2004). Activation of the PI3K pathway was determined indirectly by assessing phosphorylation of PKB.

Both the synthetic PPAR $\gamma$  ligand, troglitazone and the naturally occurring PPAR $\gamma$  ligand 15d-PGJ<sub>2</sub> (10  $\mu$ M) induced transient phosphorylation of PKB which was detectable at 15 mins and declined back to basal levels after 60 mins (Figure 4.2A). Phosphorylation of PKB by both troglitazone and 15d-PGJ<sub>2</sub> was also shown to be concentration dependent at 30 mins, (Figure 4.2B). Troglitazone responses were detected following 1-100  $\mu$ M, whilst 15d-PGJ<sub>2</sub> responses were detectable following 10-100  $\mu$ M. Subsequent experiments were performed with 10  $\mu$ M of either troglitazone or 15d-PGJ<sub>2</sub>. The competitive PI3K inhibitor LY294002 completely abolished the effects of both troglitazone and 15d-PGJ<sub>2</sub> on PKB phosphorylation (Figure 4.2C and 4.2D respectively). As a comparison, I also investigated the effects

of troglitazone and 15d-PGJ<sub>2</sub> on phosphorylation of ERK since this has also been reported to be downstream of PPAR $\gamma$  in other settings (Takeda et al., 2001) and as shown in Chapter 3 plays a role in induction of COX-2. Troglitazone treatment resulted in phosphorylation of ERK 1/2 MAPKs, an event associated with activation of these enzymes (Figure 4.2E). Furthermore, PD98059, a MEK inhibitor (Alessi et al., 1995), attenuated troglitazone induced ERK activation (Figure 4.2E). 15d-PGJ<sub>2</sub> did not stimulate phosphorylation of ERK (Figure 4.2A)



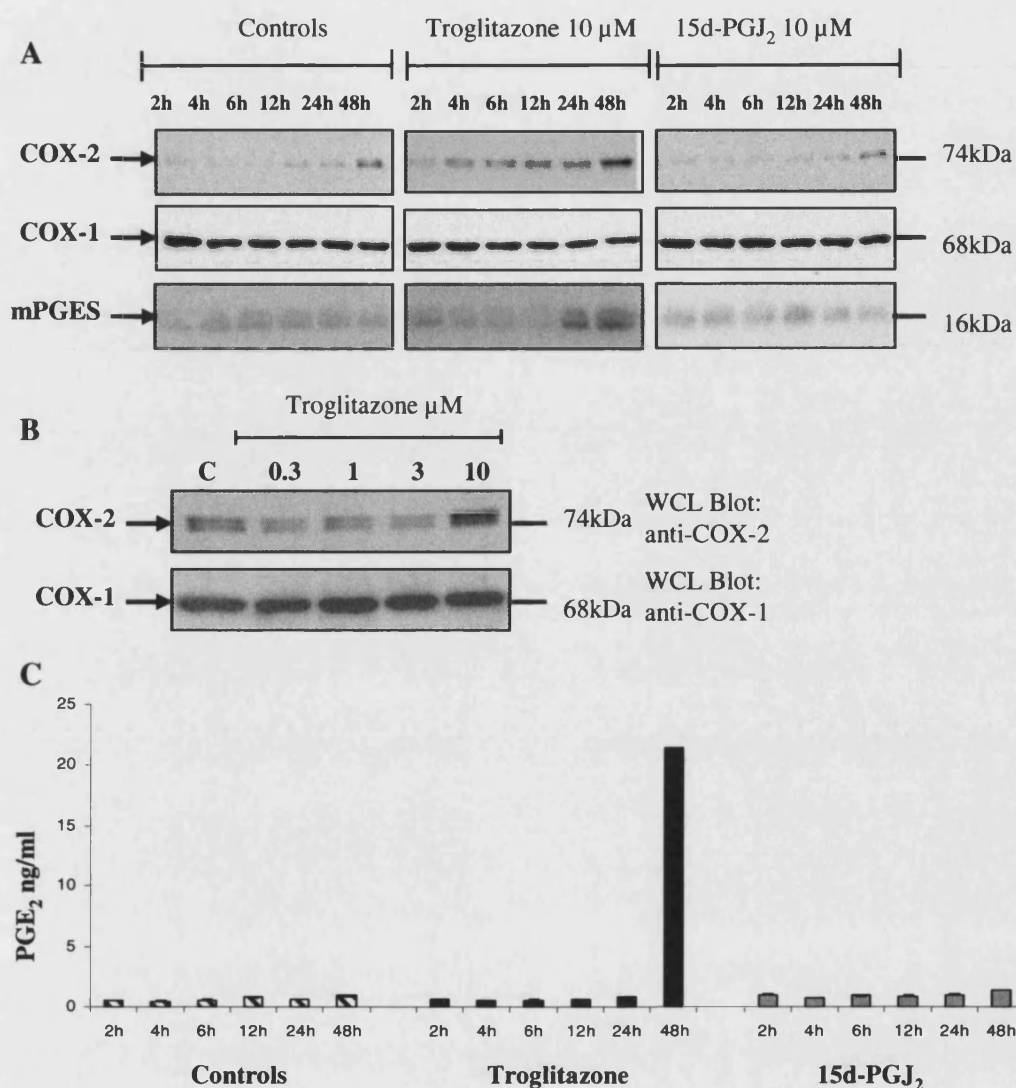
**Figure 4.2 Troglitazone stimulates PI3K and MEK dependent phosphorylation of PKB and ERK respectively, whereas 15d-PGJ<sub>2</sub> only stimulates PI3K dependent phosphorylation of PKB.**

$1.2 \times 10^6$  growth arrested, confluent A549 cells were stimulated (for the indicated times) with either troglitazone or 15d-PGJ<sub>2</sub> (10 μM) (A); or with varying concentrations of troglitazone or 15d-PGJ<sub>2</sub> for 30 mins (B); or pretreated with LY294002 10 μM for 30 mins or with PD98059 20 μM for 60 mins followed by stimulation with troglitazone (10 μM) (C and E); or pretreated with LY294002 10 μM for 30 mins followed by stimulation with 15d-PGJ<sub>2</sub> (10 μM) (D). Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and resolved by SDS-PAGE, Immunoblotting was carried out, as described in *Methods and Materials*, with a phospho-specific ERK Ab with an affinity for phosphorylated Thr<sup>202</sup>/Tyr<sup>204</sup> sites (middle panel A and upper panel C) or a phospho-specific PKB Ab with affinity for the active Ser<sup>473</sup>-phosphorylated form of PKB (upper panels A, B, D and E). Proteins were visualized with ECL. Blots were stripped and reprobbed with anti-PKB or anti-ERK Ab to verify equal loading and efficiency of protein transfer (lower panels). Data is representative of three separate experiments.

**Effect of PPAR $\gamma$  on COX-2 expression and activity.**

The effects of troglitazone and 15d-PGJ<sub>2</sub> on COX-2 expression were examined over times ranging from 2 hours to 48 hours post-stimulation in A549 cells. Untreated A549 cells had barely detectable levels of COX-2 expression (Figure 4.3A, left panel). However, after 48 hours in the experimental environment COX-2 expression was more prominent but this did not correlate with detectable basal production of PGE<sub>2</sub>, a marker of COX-2 activity (Figure 4.3A, middle panel). Troglitazone (10  $\mu$ M) induced COX-2 protein expression in a time dependent manner from 4 hours to 48 hours while COX-1 levels were unaltered (Figure 4.3A). In addition, troglitazone induced COX-2 expression was concentration-dependent at 6 hours (Figure 4.3B). The increase in COX-2 expression in response to troglitazone was accompanied by a marked increase in production of PGE<sub>2</sub> at 48 hours (Figure 4.3C). Troglitazone stimulated expression of mPGES, with kinetics similar to those observed for COX-2 induction (Figure 4.3A). However, 15d-PGJ<sub>2</sub> (10  $\mu$ M) was unable to alter expression of COX-2, mPGES or PGE<sub>2</sub> production above levels seen in time matched controls.





**Figure 4.3** Troglitazone, but not 15d-PGJ<sub>2</sub>, induces COX-2 and mPGES expression and PGE<sub>2</sub> production in a time-dependent manner in A549 cell line.

$1.2 \times 10^6$  growth arrested confluent A549s were stimulated at 37°C with either troglitazone (10  $\mu$ M) or 15d-PGJ<sub>2</sub> (10  $\mu$ M) over a time course (2-48 hrs) (A and C). Controls were carried out for each time point.  $1.2 \times 10^6$  growth arrested confluent A549s were stimulated at 37°C for 6 hours with increasing concentrations of troglitazone or 15d-PGJ<sub>2</sub> (B). Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25  $\mu$ g) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted, as described in *Methods and Materials*, with an anti-COX-2 Ab or anti-mPGES Ab (A and B; upper and lower panels respectively). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-COX-1 Ab to verify equal loading and efficiency of protein transfer (middle panels). Prior to lysing of cells, supernatants were collected and analysed for PGE<sub>2</sub> production by ELISA (C). Samples were assayed in duplicate and results are expressed as mean. Data is representative of three separate experiments.

### Evaluation of the involvement of PPAR $\gamma$ in Troglitazone induced COX-2

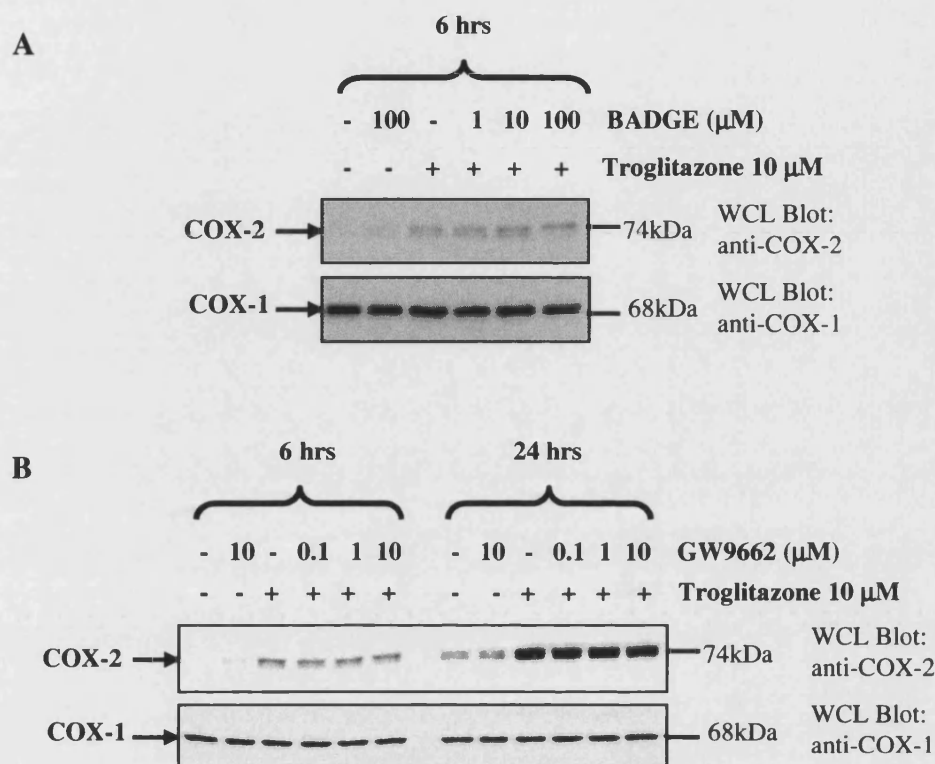
I next investigated whether induction of COX-2 by troglitazone was mediated via PPAR $\gamma$  by use of the two selective PPAR $\gamma$  antagonists, GW9662 and bisphenol A diglycidyl ether (BADGE). Neither BADGE (1–100  $\mu$ M) nor the irreversible PPAR $\gamma$  antagonist, GW9662 (Leesnitzer et al., 2002) (0.1– 10  $\mu$ M), were able to inhibit troglitazone induced COX-2 expression at 6 hours or 24 hours (Figure 4.4A and 4.4B).

### Effect of selective PPAR agonists on COX-2 expression.

To elucidate whether the disparate effects seen with the two ligands were due to effects on other isoforms of PPAR, three highly selective PPAR agonists were utilised. Neither GW262570 (PPAR $\gamma/\alpha$  agonist; Figure 4.5A) or BRL49653 (PPAR $\gamma$  agonist; Figure 4.5B) induced COX-2 expression. L-165041 (PPAR $\delta$  agonist; Figure 4.5C) showed slight induction of COX-2 at high concentration (10–30  $\mu$ M). GW262570 has preferred selectivity towards PPAR $\gamma$ , BRL49653 is also a PPAR $\gamma$  selective agonist with an EC<sub>50</sub> of 0.04  $\mu$ M (Lehmann et al., 1995) whereas L-165041 is PPAR $\delta$  selective at low micromolar range EC<sub>50</sub> values are shown in the table below (Table 4.1).

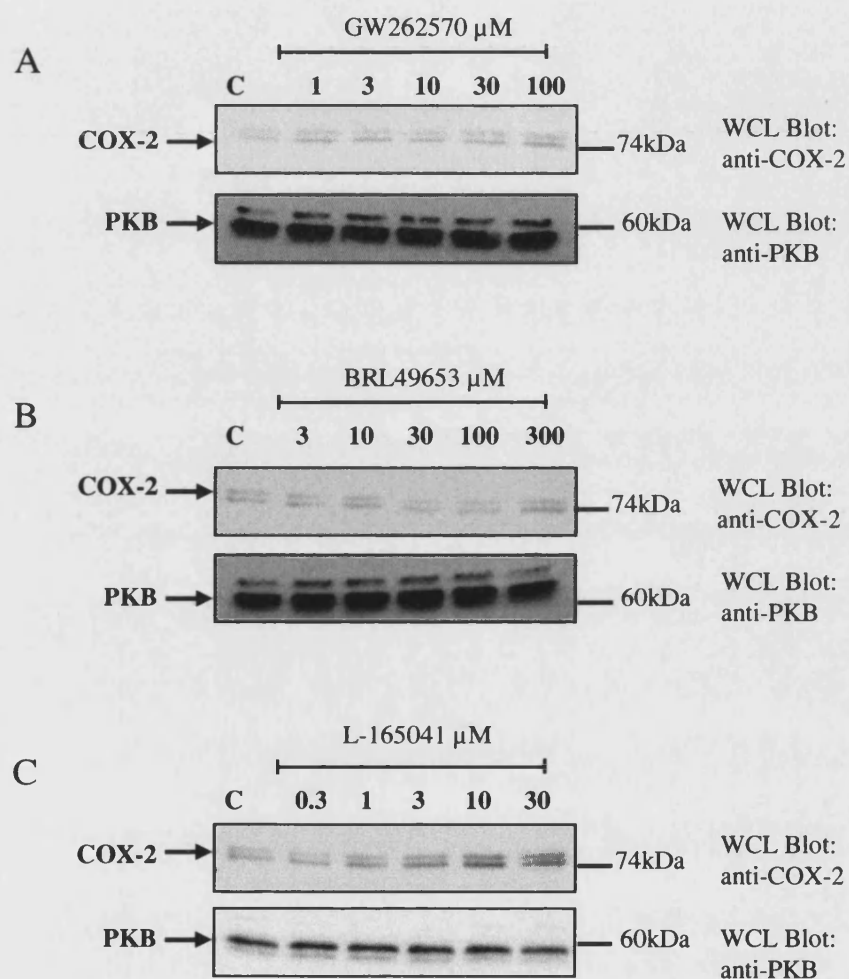
	EC <sub>50</sub> $\mu$ M		
	PPAR $\alpha$	PPAR $\gamma$	PPAR $\delta$
GW262570	0.4	0.0003	-
BRL49653 (Rosiglitazone)	-	0.04	-
L-165041	10	5.5	0.5

**Table 4.1.** EC<sub>50</sub> values of three PPAR agonists obtained from cell based assays (obtained from Novartis Horsham Research Centre courtesy of Dr. Paul Whittaker, Novartis Horsham Research Centre, U.K.).



**Figure 4.4. Troglitazone induced COX-2 expression is insensitive to two PPAR $\gamma$  antagonist.**

$1.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 1 hour with PPAR $\gamma$  antagonist, BADGE (1-100  $\mu\text{M}$ ) (A) and for 30 mins with PPAR $\gamma$  antagonist, GW9662 (0.1-10  $\mu\text{M}$ ) (B) prior to stimulation with troglitazone (10  $\mu\text{M}$ ) for 6 hrs (unless otherwise stated). Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25  $\mu\text{g}$ ) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-COX-2 Ab (upper panels). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-COX-1 Ab to verify equal loading and efficiency of protein transfer (lower panels). Data is representative of three separate experiments.



**Figure 4.5** Effect of selective PPAR agonists on COX-2 regulation.

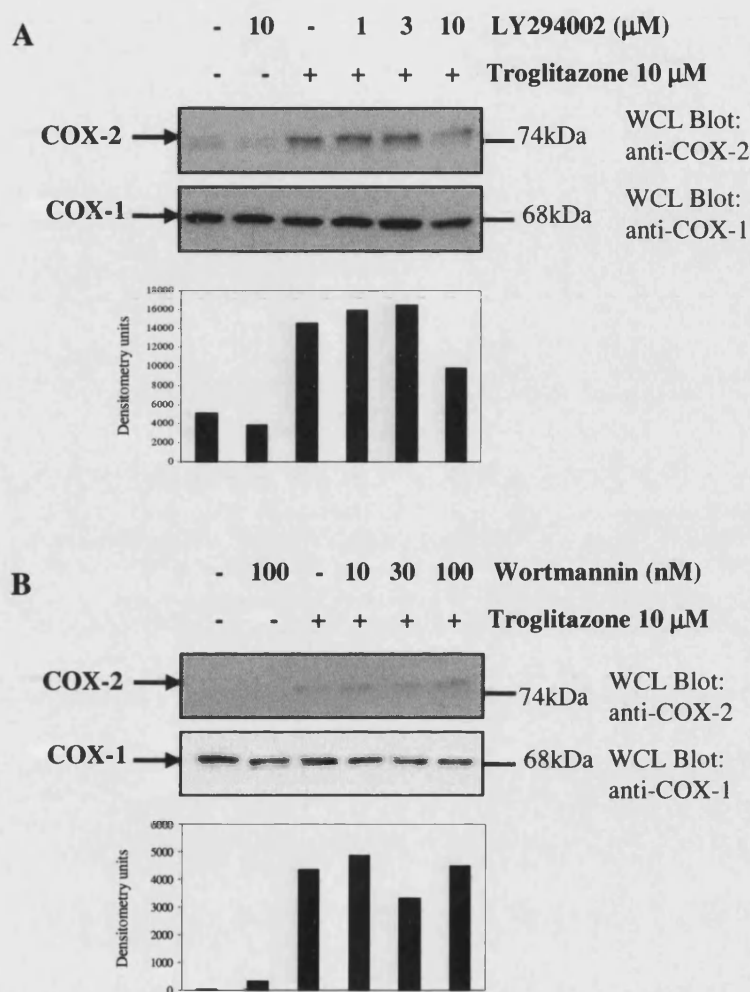
$3.2 \times 10^6$  growth arrested confluent A549 cells were treated with GW262570 (1  $\mu\text{M}$ -100  $\mu\text{M}$ ) (A), BRL49653 (3  $\mu\text{M}$  - 300  $\mu\text{M}$ ) (B) or L-165041 (0.3  $\mu\text{M}$ - 30  $\mu\text{M}$ ) (C) for 6 hrs. Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25  $\mu\text{g}$ ) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-COX-2 Ab (upper panels). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer (lower panels). Data is representative of more than three separate experiments.

### **Involvement of PI3K pathway in Troglitazone induced COX-2 expression**

Two PI3K inhibitors LY294002 and wortmannin showed differential effects on troglitazone induced COX-2 expression (Figure 4.6A and 4.6B). LY294002 showed a 33% inhibition of troglitazone induced COX-2 expression at 10  $\mu$ M (Figure 4.6A). In comparison wortmannin showed no ability to attenuate the troglitazone induced response (Figure 4.6B). Neither inhibitor affected COX-1 expression.

### **Effects of ERK and p38 MAPK and PKC inhibitors on Troglitazone induced COX-2 expression**

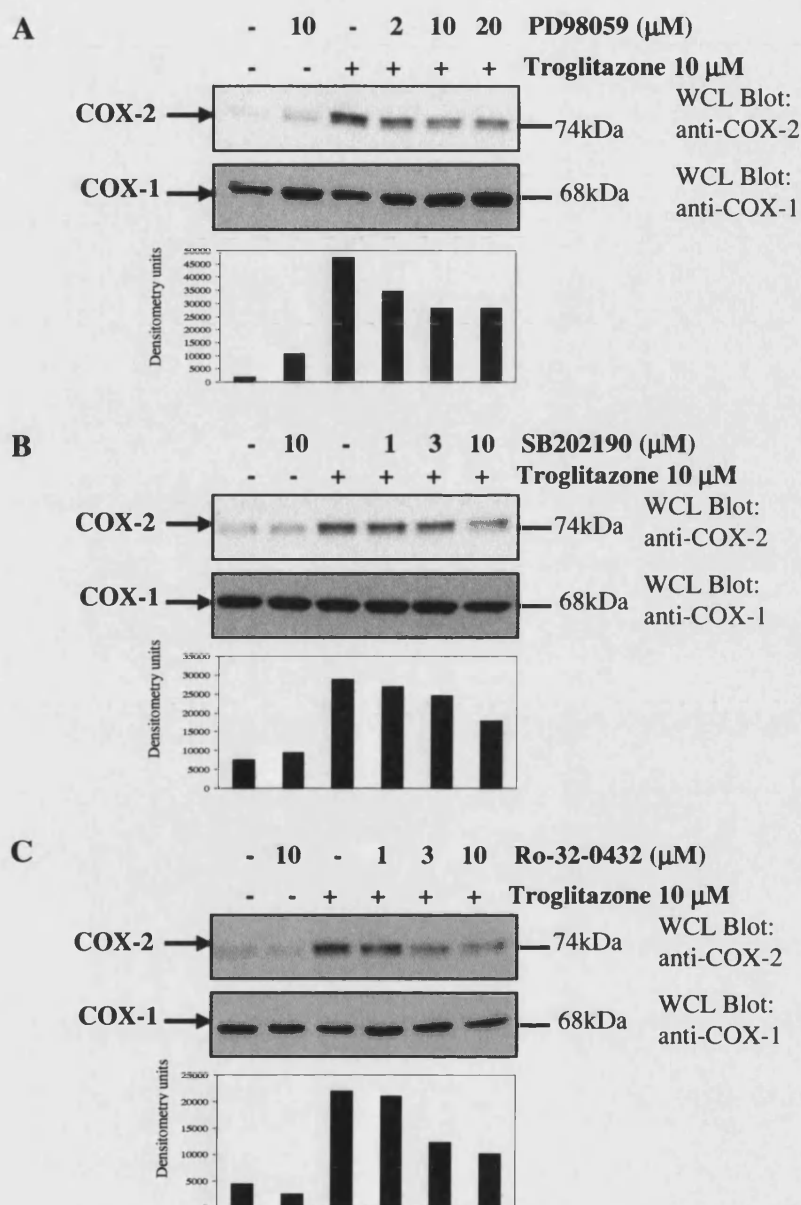
The MEK inhibitor, PD98059, showed a concentration-dependent inhibition of troglitazone-induced COX-2 (Figure 4.7A) with 40% inhibition seen at 10-20  $\mu$ M. SB202190 also showed a concentration dependent inhibition of troglitazone induced COX-2 expression (Figure 4.7B), with a 38% inhibition seen at 10  $\mu$ M. Furthermore, the broad-spectrum PKC inhibitor Ro-32-4032 showed concentration dependent inhibition of COX-2 expression (Figure 4.7C), with a 54 % inhibition at 10  $\mu$ M. As expected none of the inhibitors assessed affected COX-1 expression. These results suggest that troglitazone-induced COX-2 expression is mediated independently of PPAR $\gamma$ , but is partially dependent on the PI3K, ERK and p38 MAPK and PKC pathways.



**Figure 4.6. Effect of PI3K inhibitors on Troglitazone induced COX-2 expression**

$1.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 30 mins with PI3K inhibitor, LY294002 (1-10 μM) (A) and for 10 mins with another PI3K inhibitor wortmannin (10-100 nM) (B) prior to stimulation with troglitazone (10 μM) for 6 hrs. Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25 μg) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-COX-2 Ab (upper panels). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-COX-1 Ab to verify equal loading and efficiency of protein transfer (lower panels). Densitometry was carried out on COX-2 blots in order to quantify results and values are graphically represented below the respective blot. Data is representative of three separate experiments.





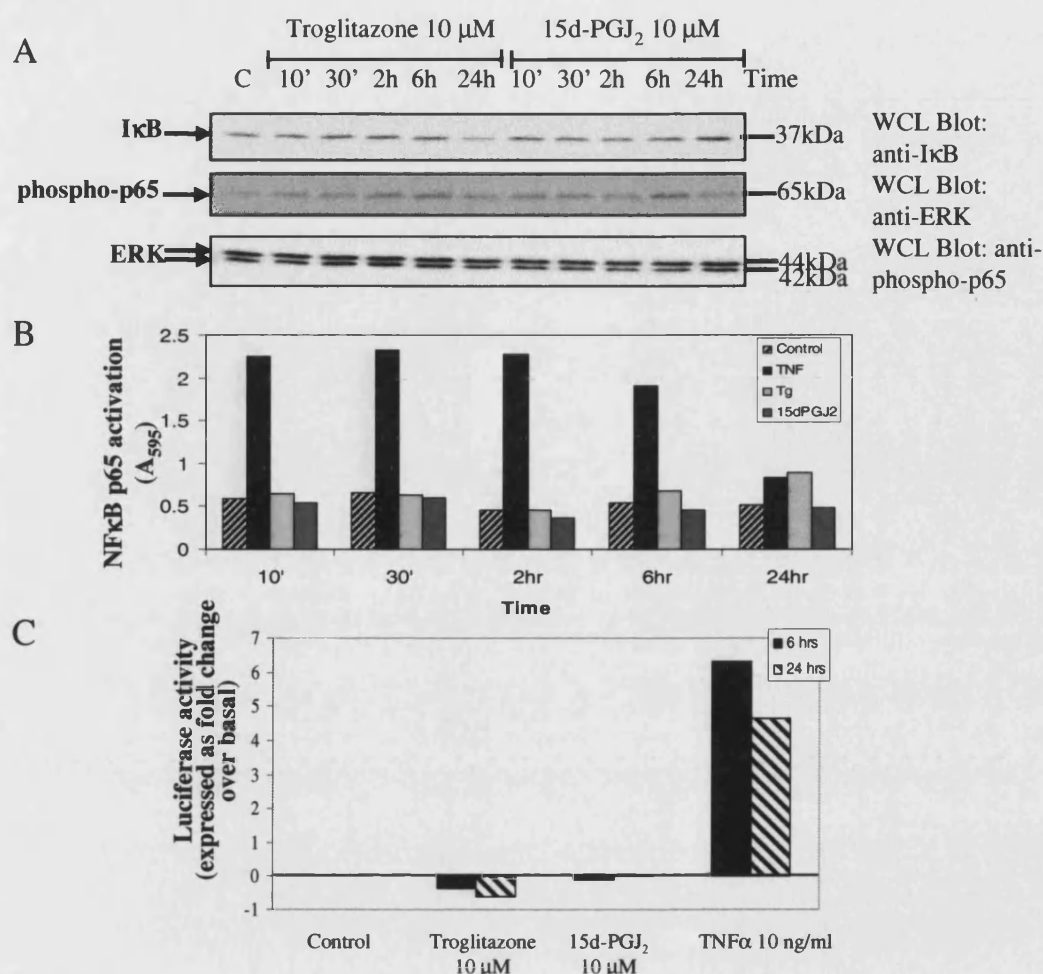
**Figure 4.7 Effect of ERK, p38 MAPK and PKC inhibitors on troglitazone induced COX-2 expression.**

$1.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 1 hr with MEK inhibitor, PD98059 (2-20 μM) (A); for 1 hour with the p38 inhibitor SB202190 (1-10 μM) (B) and for 30 mins with the PKC inhibitor Ro-32-0432 (1-10 μM) (C), prior to stimulation with troglitazone (10 μM) for 6 hrs. Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25 μg) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-COX-2 Ab (upper panels). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-COX-1 Ab to verify equal loading and efficiency of protein transfer (lower panels). Densitometry was carried out on COX-2 blots in order to quantify results and values are graphically represented below the respective blot. Data is representative of three separate experiments.

**Effects of PPAR $\gamma$  agonists on NF $\kappa$ B activation.**

Since troglitazone markedly induced COX-2 expression and PGE<sub>2</sub> production at 24 to 48 hours (observed in Figure 4.3), it was next investigated whether the NF $\kappa$ B pathway was involved in the induction of COX-2 expression. I first assessed NF $\kappa$ B activation indirectly by monitoring degradation of cytosolic I $\kappa$ B, which under resting conditions is bound to and inhibits NF $\kappa$ B. Thus induction of the classical NF $\kappa$ B pathway firstly requires degradation of I $\kappa$ B. Neither troglitazone nor 15d-PGJ<sub>2</sub> induced I $\kappa$ B degradation at early phase time points (up to 2 hours) (Figure 4.8A; upper panels). Modest I $\kappa$ B degradation was seen with troglitazone at 24 hours (Figure 4.8A; upper panels), but neither troglitazone nor 15d-PGJ<sub>2</sub> stimulated phosphorylation of p65. NF $\kappa$ B activation was next investigated by monitoring activation of the binding ability of NF $\kappa$ B p65 to consensus oligonucleotide sequences by means of the TransAM NF $\kappa$ B p65 kit. Once again, TNF $\alpha$  induced marked activation of NF $\kappa$ B p65 from 10 mins through to 2 hours (Figure 4.8B). Troglitazone induced slight activation of NF $\kappa$ B at 24 hours (Figure 4.8B), which was consistent with the minimal degradation of I $\kappa$ B observed at 24 hours. 15d-PGJ<sub>2</sub> had no effect on NF $\kappa$ B p65 activation (Figure 4.8B). A final method for assessing NF $\kappa$ B activity was to use an A549 cell line stably transfected with NF $\kappa$ B luciferase gene reporter construct. TNF $\alpha$  greatly induced NF $\kappa$ B luciferase gene reporter activity at both 6 hrs and 24 hrs (Figure 4.8C). In comparison, neither troglitazone nor 15d-PGJ<sub>2</sub> activated NF $\kappa$ B luciferase gene reporter activity above basal levels at 6 or 24 hours (Figure 4.8C).

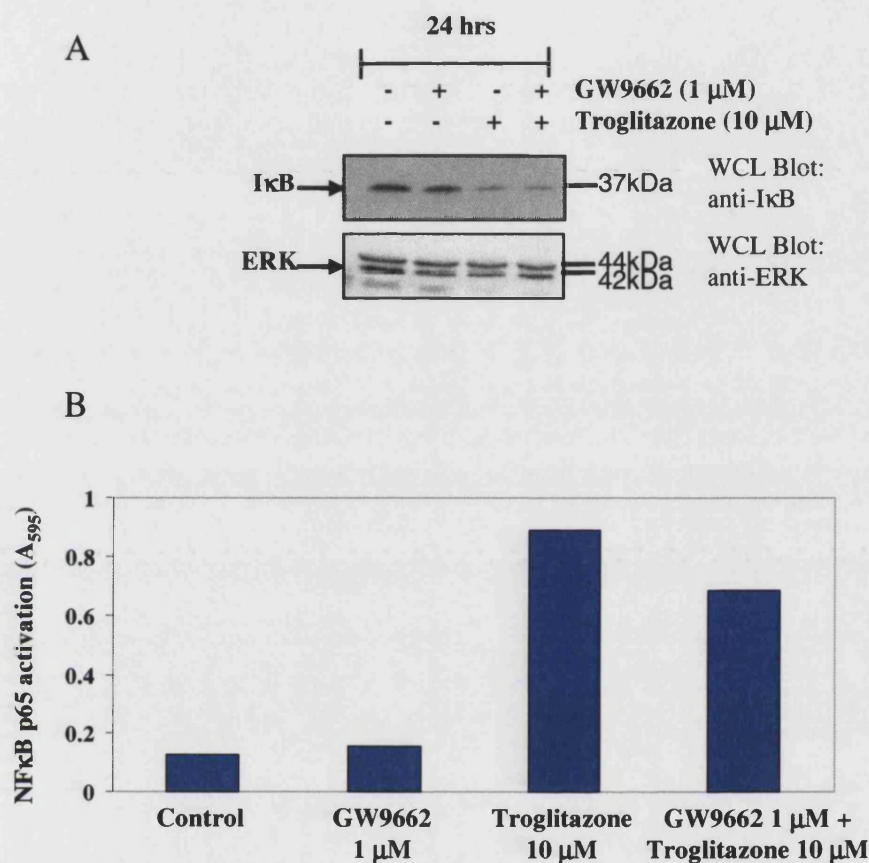




**Figure 4.8** Neither troglitazone nor 15d-PGJ<sub>2</sub> induced, degradation of IκB, NFκB p65 phosphorylation or activation or NFκB gene reporter activity in comparison to TNFα.

$3.2 \times 10^6$  growth arrested confluent A549 cells were stimulated with either troglitazone (10 μM) or 15d-PGJ<sub>2</sub> (10 μM) (A) over a time course (10 mins -24 hrs). Nuclear and cytosolic proteins were extracted as described in *Methods and Materials*. Proteins were quantified and cytosolic protein samples were boiled with 5 X sample buffer. Protein (25 μg) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-IκB Ab (upper panels, A) or an anti-phospho-p65 Ab (middle panels, A). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-ERK Ab to verify equal loading and efficiency of protein transfer (lower panels, A). Nuclear extracts (7.5 μg) from the same experiments were assayed for NFκB p65 activation/DNA binding by use of a TransAM™ NFκB p65 kit (B). Samples were assayed in duplicate and results are expressed as mean NF-κB p65 activation (A<sub>595</sub>). Data is representative of three separate experiments. C-  $0.2 \times 10^6$  growth arrested, confluent A549-κBtkluc cells (A549 cells that have been stably transfected with the NF-κB luciferase gene reporter construct) were stimulated with troglitazone (10 μM), 15d-PGJ<sub>2</sub> (10 μM) or TNFα 10 ng/ml (as a positive control) for 6 and 24 hours. Promega Steady-Glo luciferase buffer was added to cells and left at room temperature for 10 mins. Lysates were then mixed and 200 μl sample was then transferred into opaque 96-well plates and luminescence was measured on a Beckman Topcount with a 5 sec luminescence program. Results are expressed as mean luminescence ± s.e.m. Data is representative of more than three separate experiments.

Next it was investigated whether the modest I $\kappa$ B degradation and NF $\kappa$ B p65 activation induced by troglitazone at 24 hours was dependent on PPAR $\gamma$ . Surprisingly, the troglitazone-induced degradation of I $\kappa$ B and p65 activation observed at 24 hours were not inhibited by pre-treatment with PPAR $\gamma$  antagonist GW9662 (Figure 4.9A and 4.9B), thus suggesting that troglitazone can induce I $\kappa$ B degradation independently of PPAR $\gamma$ .

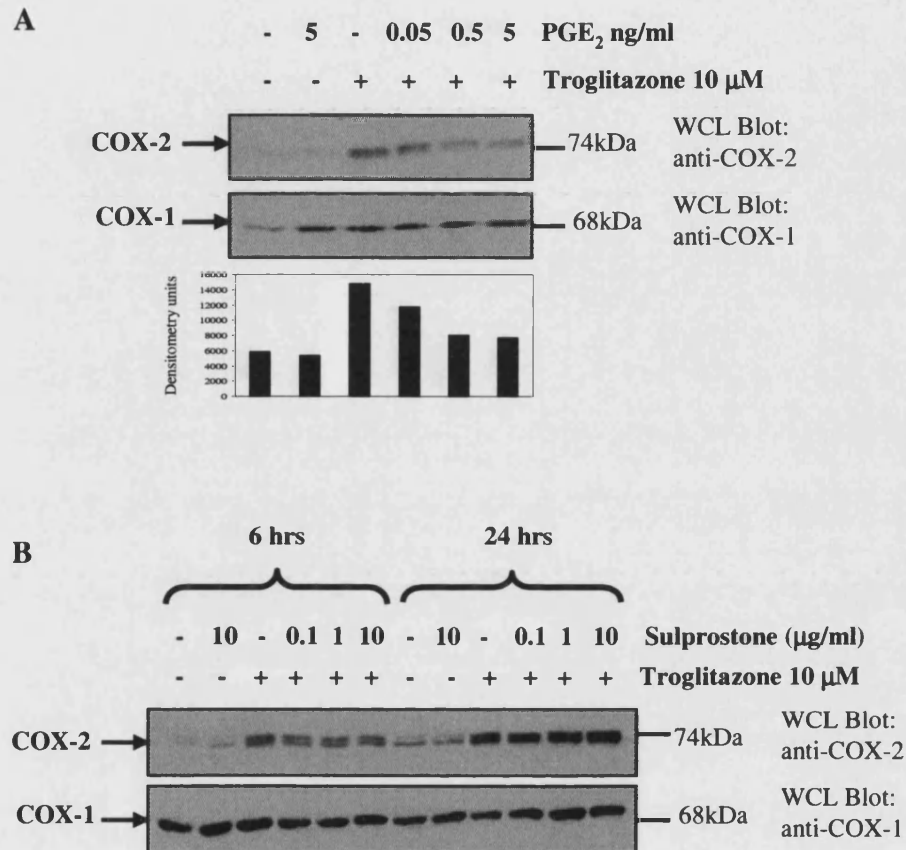


**Figure 4.9 Minimal activation of NFκB pathway by Troglitazone at 24 hours is independent of PPARγ**

$3.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 30 mins with GW9662 (1  $\mu$ M) prior to stimulation with troglitazone (10 $\mu$ M) for 24 hours. Nuclear and cytosolic proteins were extracted as described in *Methods and Materials*. Proteins were quantified and cytosolic protein samples were boiled with 5 X sample buffer. 25  $\mu$ g of protein was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-IκB Ab (upper panel, A). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-ERK Ab to verify equal loading and efficiency of protein transfer (lower panel, A). 7.5  $\mu$ g of nuclear extracts from the same experiments were assayed for NFκB p65 activation/DNA binding by use of a TransAM™ NFκB p65 kit (B). Samples were assayed in duplicate and results are expressed as mean NFκB p65 activation (A<sub>595</sub>). Data is representative of three separate experiments.

### **Effect of PGE<sub>2</sub> and PGE<sub>2</sub> analogue on troglitazone induced COX-2 expression**

Previous studies have shown that the COX-2 product, PGE<sub>2</sub>, can negatively feedback to regulate COX-2 expression (Akarasereenont et al., 1999). Furthermore vast amounts of PGE<sub>2</sub> were produced following stimulation with troglitazone but only at late time point (48 hrs). Thus this next section investigates whether the huge increase in PGE<sub>2</sub> plays a feedback role and thus may regulate troglitazone induced COX-2. The concentration of PGE<sub>2</sub> used was 0.05ng/ml to 5ng/ml since the level of PGE<sub>2</sub> produced by stimulated cells is within these concentrations. PGE<sub>2</sub> inhibited troglitazone induced COX-2 expression in a concentration dependent manner (Figure 4.10A) at 6 hours, with 47 % inhibition observed at 5 ng/ml. PGE<sub>2</sub> can act on all four EP receptor subtypes. Next it was investigated whether an EP<sub>3</sub> selective agonist, sulprostone, would have the same effect as PGE<sub>2</sub>. Sulprostone showed little inhibition of troglitazone induced COX-2 at 6 hours and no inhibition at 24 hours (Figure 4.10B).



**Figure 4.10. Regulation of troglitazone induced COX-2 expression by PGE<sub>2</sub> and a PGE<sub>2</sub> analogue.**

$1.2 \times 10^6$  growth arrested confluent A549 cells were co-treated with PGE<sub>2</sub> (0.05-5 ng/ml) and troglitazone (10  $\mu$ M) (A); or with sulprostone (0.1-10  $\mu$ g/ml) and troglitazone (10  $\mu$ M) (B) for 6 hours (unless otherwise stated). Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25  $\mu$ g) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-COX-2 Ab (upper panels). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-COX-1 Ab to verify equal loading and efficiency of protein transfer (lower panels). Densitometry was carried out on COX-2 blots in order to quantify results and values are graphically represented below the respective blot. Data is representative of three separate experiments.

### Effect of Troglitazone and 15d-PGJ<sub>2</sub> on gene activation assessed by microarray analysis

RNA microarray analysis was utilised to investigate the regulation of genes by troglitazone and 15d-PGJ<sub>2</sub>. Table 4.2 shows that 15d-PGJ<sub>2</sub> up-regulated only 1 gene and downregulated 9 genes, 6 hours post-stimulation. In comparison, troglitazone upregulated 31 genes and downregulated 16 genes at the same time point (Table 4.3). The only similarity in gene regulation by the two ligands was that both inhibited ribosomal S2 protein and heat shock protein. This data suggests that in addition to disparate effects on signalling pathways, troglitazone and 15d-PGJ<sub>2</sub> manifest different gene expression patterns both in number and the types of genes observed. Surprisingly COX-2 expression was not found to be regulated by troglitazone, using the criteria for selection.

Description	Average Control	Average 15d-PGJ <sub>2</sub>	Fold Change
<b>Upregulated</b>			
acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	0.685	1.478	2.157
<b>Downregulated</b>			
fasciculation and elongation protein zeta 2 (zygin II)	1.084	0.424	-2.559
TRAF-interacting protein I-TRAF mRNA, complete cds.	1.585	0.707	-2.242
heat shock 70kD protein 9B (mortalin-2)	2.107	0.953	-2.211
clone FLB4941 PRO1292 mRNA, complete cds.	1.813	0.820	-2.210
NCK-associated protein 1 (NCKAP1), mRNA.	1.847	0.848	-2.178
ribosomal protein S2	1.510	0.721	-2.095
complement C1r-like proteinase precursor, (LOC51279), mRNA.	1.481	0.719	-2.059
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10 (GalNAc-T10)	1.762	0.867	-2.033
chromosome 1 open reading frame 28	1.511	0.747	-2.022

**Table 4.2. Microarray analysis comparing genes upregulated or downregulated following treatment with 15d-PGJ<sub>2</sub>.**  $8.8 \times 10^6$  growth arrested confluent A549 cells stimulated with 15-dPGJ<sub>2</sub> (10  $\mu$ M) for 6 hours. RNA was isolated using Trizol, RNA quantified and 100ng per well was run on RNA chips on Agilent 2100 Bioanalyzer. RNA samples were processed and analysed as described in *Methods and Materials*.



Description	Average Control	Average Troglitazone	Fold Change
<b>Upregulated</b>			
activating transcription factor 4 (tax-responsive enhancer element B67) (ATF4), mRNA.	0.840	1.951	2.321
dual specificity phosphatase 1	0.810	1.822	2.250
basic helix-loop-helix domain containing, class B, 2	0.799	1.664	2.081
methylene tetrahydrofolate dehydrogenase (NAD <sup>+</sup> dependent), methenyltetrahydrofolate cyclohydrolase (MTHFD2), nuclear gene encoding mitochondrial protein, mRNA.	0.934	2.109	2.258
3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), mRNA.	0.940	1.889	2.009
activating transcription factor 3 (ATF3), mRNA.	0.746	1.947	2.608
phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2), mRNA.	0.759	1.541	2.032
interleukin 8 (IL8), mRNA.	0.981	2.434	2.483
HIF-1 responsive RTP801	0.932	2.145	2.302
stanniocalcin 2, clone MGC:1881, mRNA, complete cds.	0.876	2.123	2.423
sodium channel, nonvoltage-gated 1 alpha (SCNN1A), mRNA.	0.645	1.427	2.210
transcription elongation factor A (SII), 2 (TCEA2), mRNA.	0.633	1.323	2.090
mevalonate kinase (mevalonic aciduria) (MVK), mRNA.	0.635	1.439	2.265
hypothetical protein FLJ10374 (FLJ10374), mRNA.	0.796	1.616	2.031
S100 calcium-binding protein P (S100P), mRNA.	0.909	1.837	2.022
KIAA0590 gene product (KIAA0590), mRNA.	0.698	1.483	2.124
Asparagine synthetase (ASNS), mRNA.	0.929	2.365	2.545
zinc finger DAZ interacting protein 3	0.860	1.763	2.051
delta sleep inducing peptide, immunoreactor	1.017	3.297	3.243
inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	0.977	1.959	2.006
Adipose differentiation-related protein, clone MGC: 10598, mRNA, complete cds.	0.698	2.317	3.319
p8 protein homolog (COM1) mRNA, complete cds.	0.822	1.914	2.329
vascular endothelial growth factor mRNA, complete cds.	0.863	1.777	2.060
lipin 1	0.781	1.949	2.497
DNA sequence from clone RP3-408B20 on chromosome 6	0.820	1.858	2.265
cytochrome b reductase 1	0.592	1.203	2.030
protein kinase domains containing protein similar to phosphoprotein C8FW (LOC57761), mRNA.	0.854	2.433	2.849
TGFB inducible early growth response 2	0.866	2.208	2.549
Fanconi anemia, complementation group F (FANCF), mRNA.	0.791	1.626	2.055
hypothetical protein MGC4054 (MGC4054), mRNA.	0.728	1.481	2.033
aquaporin 3	0.704	1.640	2.331
<b>Downregulated</b>			
gap junction protein, alpha 7, 45kD (connexin 45) (GJA7), mRNA.	1.472	0.586	-2.513
bromodomain adjacent to zinc finger domain, 1A	1.222	0.496	-2.466
cDNA FLJ11304 fis, clone PLACE1009997, weakly similar to Rattus norvegicus A-kinase anchoring protein AKAP 220 mRNA.	1.224	0.500	-2.448
ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1), mRNA.	0.995	0.417	-2.384
ATP-binding cassette transporter 1 (ABCA1) mRNA, complete cds.	1.092	0.475	-2.301
glutaminase C mRNA, complete cds.	2.005	0.887	-2.262

heat shock 70kD protein 9B (mortalin-2)	2.107	0.944	-2.233
hypothetical protein FLJ12701 (FLJ12701), mRNA.	1.163	0.527	-2.207
DNA sequence from clone RP3-406P24 on chromosome 6 Contains a thioredoxin-like pseudogene, 2 CpG islands, ESTs, STSs and GSSs	1.568	0.712	-2.201
DnaJ (Hsp40) homolog, subfamily B, member 4	1.114	0.507	-2.197
KIAA0924 protein (KIAA0924), mRNA.	1.380	0.647	-2.133
disabled (Drosophila) homolog 2 (mitogen-responsive phosphoprotein) (DAB2), mRNA.	1.832	0.898	-2.042
3-hydroxyisobutyryl-Coenzyme A hydrolase	1.600	0.793	-2.017
ribosomal protein S2	1.510	0.749	-2.015
cDNA: FLJ21305 fis clone COL02124.	1.495	0.746	-2.005
thromboxane A2 receptor (TBXA2R), mRNA.	1.055	0.527	-2.002

**Table 4.3. Microarray analysis comparing genes upregulated or downregulated following treatment with troglitazone.**  $8.8 \times 10^6$  growth arrested confluent A549 cells stimulated with troglitazone (10  $\mu$ M) for 6 hours. RNA was isolated using Trizol, RNA quantified and 100ng per well was run on RNA chips on Agilent 2100 Bioanalyzer. RNA samples were processed and analysed as described in *Methods and Materials*.



## **4.2. Regulation of cytokine induced COX-2 expression and activity events by PPAR $\gamma$ ligands**

### **4.2.1. Background**

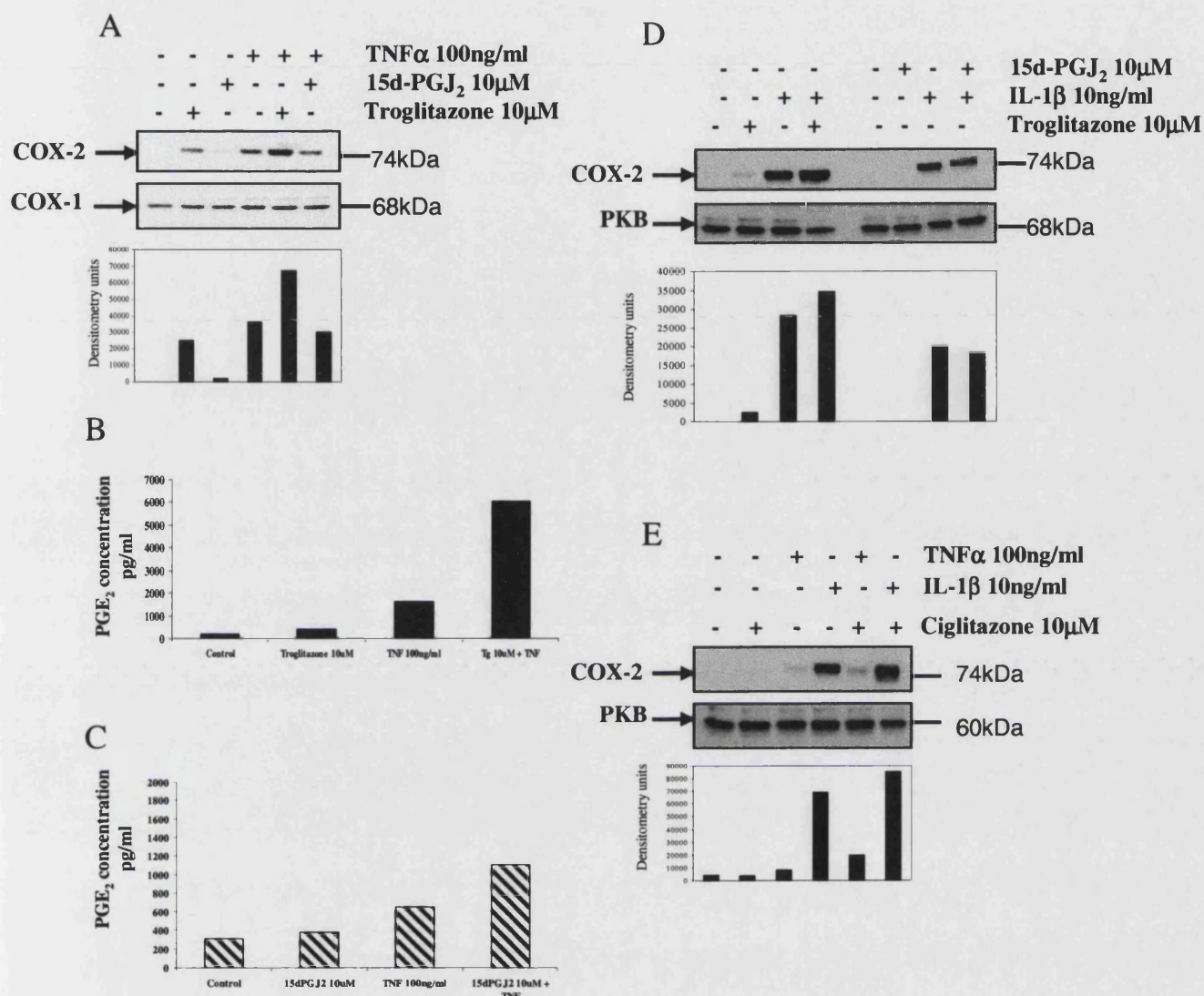
Other PPAR $\gamma$  ligands such as NSAIDs are widely used in the treatment of inflammation, and their efficacy is considered to be a result of their inhibitory effect on COX-2. NSAIDs have recently been identified as ligands of PPARs. Paik et al., reported that the NSAID, flufenamic acid, showed two opposing effects on COX-2 expression; it induces COX-2 expression in colonic epithelial cells and macrophages; conversely, it inhibits TNF $\alpha$  or LPS-induced COX-2 expression (Paik et al., 2000), and similar effects were also seen with 15d-PGJ<sub>2</sub>. These studies suggested that NSAIDs inhibit mitogen-induced COX-2 expression while they induce COX-2 expression (Paik et al., 2000). However the exact roles of PPAR $\gamma$  ligands on the regulation of COX-2 expression have not yet been fully recognized.

Having established that two structurally distinct PPAR $\gamma$  ligands regulate COX-2 expression differentially, the effects of these ligands on TNF $\alpha$  and IL-1 $\beta$  induced COX-2 expression and activity were next examined.

#### 4.2.2. Results

##### **Effect of PPAR $\gamma$ ligands on TNF $\alpha$ induced COX-2 and PGE $_2$ production**

In support of previous observations, TNF $\alpha$  and IL-1 $\beta$  stimulated COX-2 expression and PGE $_2$  production (previously shown in Figure 3.9). As expected, the combination of troglitazone and TNF $\alpha$  had an additive effect on the induction of COX-2 and also a synergistic effect on the PGE $_2$  production (Figure 4.11A and 4.11B). 15d-PGJ $_2$  showed a slight inhibitory effect on TNF $\alpha$ -induced COX-2 expression, but curiously it appeared to enhance TNF $\alpha$ -induced PGE $_2$  production (Figure 4.11A and 4.11C). Moreover, similar effects were seen when COX-2 expression was induced by IL-1 $\beta$  in that troglitazone was able to further enhance IL-1 $\beta$  induced COX-2 expression, whereas 15d-PGJ $_2$  showed a slight inhibitory effect on IL-1 $\beta$  induced COX-2 expression (Figure 4.11D). The effect of ciglitazone, another synthetic PPAR $\gamma$  ligand with similar structure to troglitazone, was investigated. Like troglitazone, ciglitazone was able to enhance both TNF $\alpha$  and IL-1 $\beta$  induced COX-2 expression (Figure 4.11E).

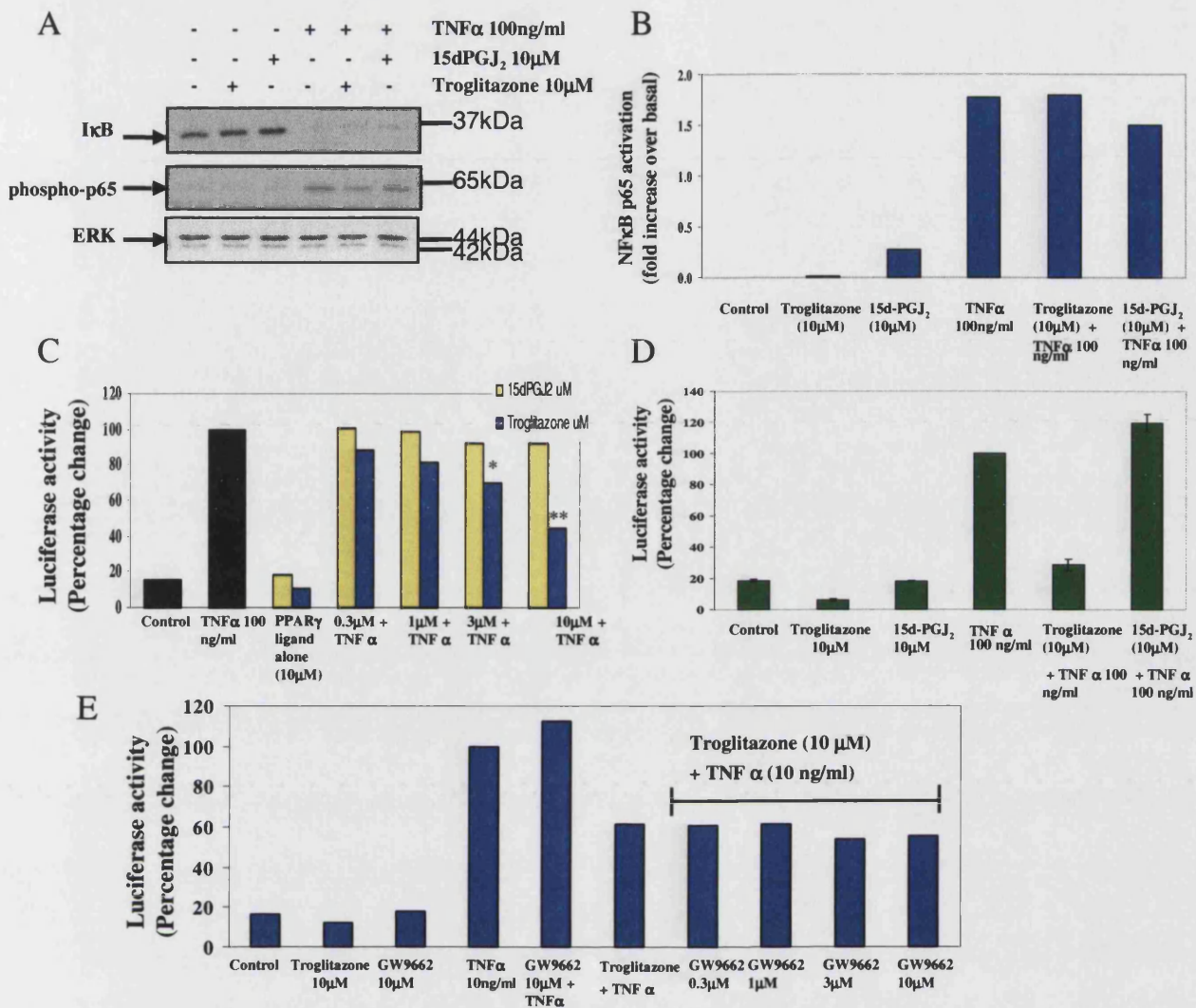


**Figure 4.11 Effect of PPAR $\gamma$  ligands on cytokine induced COX-2 expression and PGE<sub>2</sub> production.**

$1.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 1 hr with troglitazone (10 $\mu$ M), 15d-PGJ<sub>2</sub> (10  $\mu$ M) or ciglitazone (10  $\mu$ M) prior to stimulation with TNF $\alpha$  100 ng/ml or IL-1 $\beta$  10 ng/ml for 6 hrs (A, D and E). Cells were lysed with ice-cold lysis buffer, proteins extracted, quantified and boiled with 5 X sample buffer. Protein (25  $\mu$ g) was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-COX-2 Ab (A, D and E; upper panel). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer (A, D and E; lower panels). Densitometry was carried out on COX-2 blots in order to quantify results and values are graphically represented below the respective blot. Data is representative of more than three separate experiments. B and C,  $1.2 \times 10^6$  growth arrested confluent A549 cells were pretreated for 1 hr with troglitazone (10  $\mu$ M) and 15d-PGJ<sub>2</sub> (10  $\mu$ M) prior to stimulation with TNF $\alpha$  100 ng/ml for 24 hrs. Supernatants were collected and assessed for PGE<sub>2</sub> production by means of ELISA (B and C). Samples were assayed in duplicate and results are expressed as mean. Data is representative of three separate experiments.

**Effect of troglitazone and 15d-PGJ<sub>2</sub> on TNF $\alpha$  induced I $\kappa$ B degradation, NF $\kappa$ B p65 activation and NF $\kappa$ B gene reporter activity.**

Although NF $\kappa$ B activation in response to PPAR $\gamma$  agonists in a range of assays was not detected, it was next investigated whether PPAR $\gamma$  agonists could influence TNF $\alpha$  responses at the level of NF $\kappa$ B, having already demonstrated synergistic effects of troglitazone on TNF $\alpha$ -stimulated PGE<sub>2</sub> production. Pre-treatment with either PPAR $\gamma$  ligand did not inhibit TNF $\alpha$  induced I $\kappa$ B degradation, p65 phosphorylation (Figure 4.12A) or p65 activation at 30 mins (Figure 4.12B). It was next investigated whether troglitazone or 15d-PGJ<sub>2</sub> could affect TNF $\alpha$  induced NF $\kappa$ B reporter activity by means of luciferase assays. Troglitazone, but not 15d-PGJ<sub>2</sub> inhibited TNF $\alpha$  induced NF $\kappa$ B gene reporter activity in a concentration-dependent manner (Figure 4.12C) at 6 hours. Furthermore, troglitazone also inhibited TNF $\alpha$  induced NF $\kappa$ B gene reporter activity at 24 hours (Figure 4.12D). Addition of the PPAR $\gamma$  antagonist (GW9662), was unable to reverse the inhibitory effects of troglitazone on TNF $\alpha$  induced NF $\kappa$ B gene reporter activity (Figure 4.12E). This suggests that troglitazone can inhibit TNF $\alpha$ -stimulated NF $\kappa$ B gene reporter activity independently of PPAR $\gamma$ .



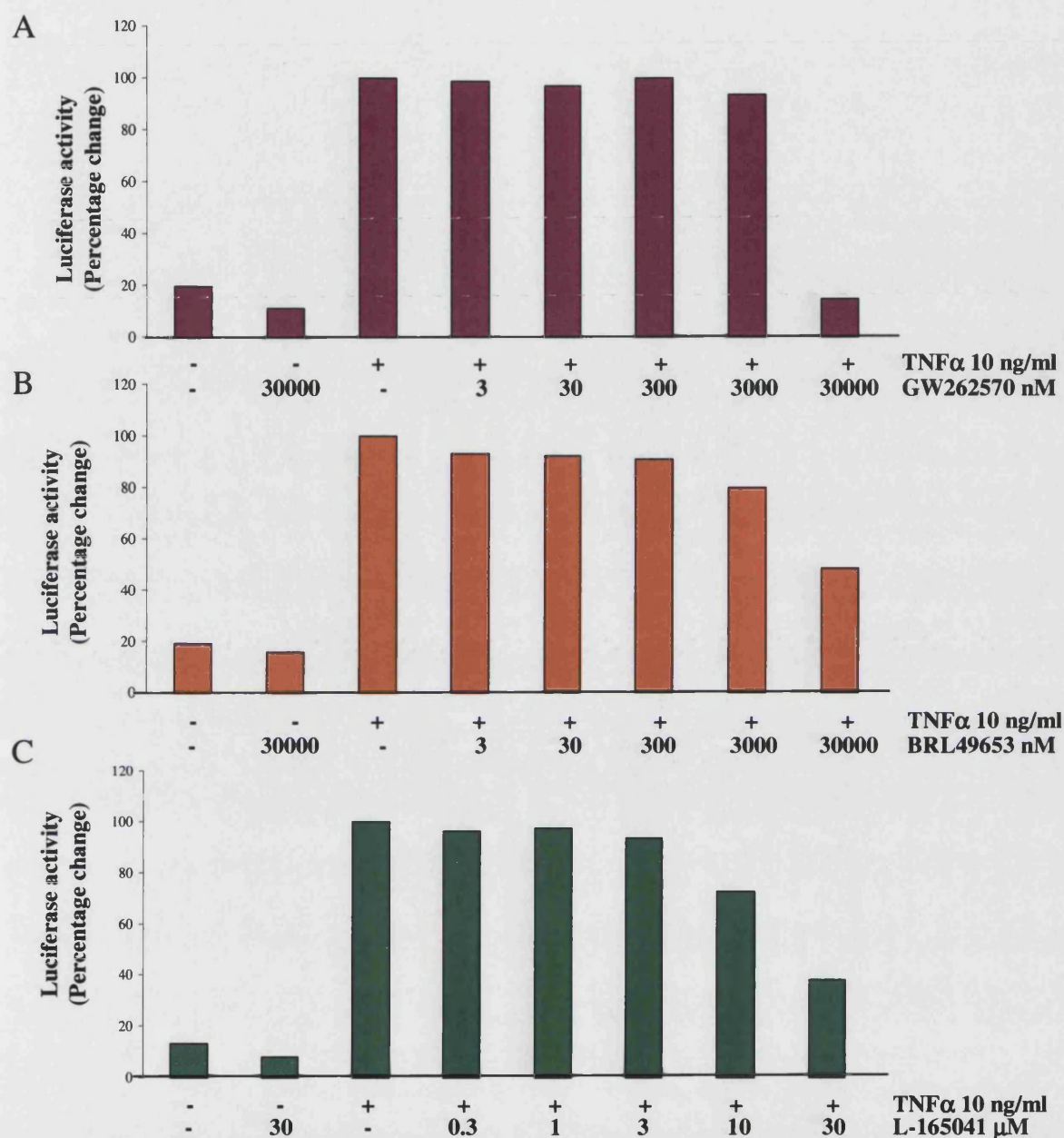
**Figure 4.12. Effect of troglitazone and 15dPGJ<sub>2</sub> on TNFα induced IκB degradation, NFκB p65 phosphorylation and activation and NFκB gene reporter activity.**

$3.2 \times 10^6$  growth arrested confluent A549 cells were pretreated with troglitazone (10 μM) or 15d-PGJ<sub>2</sub> (10 μM) for 1 hr prior to stimulation for 30 mins (A and B) and 6 hours (C and D) with TNFα 100 ng/ml. Nuclear and cytosolic proteins were extracted as described in *Methods and Materials*. Proteins were quantified and cytosolic protein samples were boiled with 5 X sample buffer. 25 μg of cytosolic proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-IκB Ab (upper panel, A) or anti-phospho-p65 Ab (middle panel, A). Proteins were visualized with ECL. Blots were stripped and reprobed with anti-ERK Ab to verify equal loading and efficiency of protein transfer (lower panels, A). 7.5 μg of nuclear extracts from the same experiment were assayed for NFκB p65 activation/DNA binding by use of a TransAM™ NFκB p65 kit (B). Results are expressed as NFκB p65 activation (fold change over basal). C- $0.2 \times 10^6$  growth arrested, confluent A549-κBtkluc cells (A549 cells that have been stably transfected with the NFκB luciferase gene reporter construct) were pretreated with troglitazone or 15d-PGJ<sub>2</sub> (with the indicated concentration) for 1 hr prior to stimulation with TNFα 100 ng/ml for 6 hours and 24 hours (C and D) or pretreated with GW9662 at the concentration indicated for 30 mins, prior to further pre-treatment with troglitazone (10 μM) or 15d-PGJ<sub>2</sub> (10 μM) for 1 hr prior to stimulation with TNFα 10 ng/ml for 6 hours (E). Promega Steady-Glo luciferase buffer was added to cells and left at room temperature for 10 mins. Lysates were then mixed and 200 μl sample was transferred into opaque 96-well plates and luminescence was measured on a Beckman Topcount with a 5 sec luminescence program. Results are expressed as percentage change. All data is representative of at least three separate experiments. \*  $P < 0.05$ , \*\*  $P < 0.001$ .

**Effect of selective PPAR agonists on TNF $\alpha$ -induced NF $\kappa$ B gene reporter activity**

Next it was investigated whether the effects of the synthetic PPAR $\gamma$  ligand troglitazone on TNF $\alpha$  induced NF $\kappa$ B activity was characteristic of other synthetic PPAR $\gamma$  ligands. GW262570, BRL49653 and L-165041 attenuated the TNF $\alpha$  induced NF $\kappa$ B gene reporter activity response, albeit at high concentrations (30  $\mu$ M), which exceed their EC<sub>50</sub> values (Figure 4.13A-C; see Table 4.1 for EC<sub>50</sub> values).





**Figure 4.13** Effect of selective PPAR agonists on TNF $\alpha$ -induced NF $\kappa$ B gene reporter activity.

$0.2 \times 10^6$  growth arrested, confluent A549- $\kappa$ Btkluc cells (A549 cells that have been stably transfected with the NF $\kappa$ B luciferase gene reporter construct) were pretreated with GW262570 (3 nM- 30  $\mu$ M) (A), BRL49653 (3 nM - 30  $\mu$ M) (B) or L-165041 (300 nM- 30  $\mu$ M) for 1 hr prior to stimulation with TNF $\alpha$  10 ng/ml for 6 hours. Promega Steady-Glo luciferase buffer was added to cells and left at room temperature for 10 mins. Lysates were then mixed and 200  $\mu$ l sample was transferred into opaque 96-well plates and luminescence was measured on a Beckman Topcount with a 5 sec luminescence program. Samples were assayed in duplicate and results are expressed as mean percentage change. Data is representative of more than three separate experiments.

### **4.3. Summary**

#### **Summary of section 4.1**

- The A549 cell line expresses PPAR $\gamma_1$  and PPAR $\gamma_2$  and expression of this receptor in this system is limited to the nucleus.
- The synthetic PPAR $\gamma$  ligand troglitazone and the endogenous ligand 15d-PGJ<sub>2</sub> both induced phosphorylation of PKB in a PI3K dependent manner.
- In addition troglitazone, but not 15d-PGJ<sub>2</sub>, activated the ERK MAPK pathway. This effect was sensitive to the MEK inhibitor, PD98059.
- Troglitazone, but not 15d-PGJ<sub>2</sub>, induced COX-2 and mPGES expression and PGE<sub>2</sub> production in a time dependent manner.
- COX-2 induction by troglitazone was insensitive to two selective PPAR $\gamma$  antagonists.
- Selective PPAR agonists showed no induction of COX-2 above basal levels with the exception of L-165041 which showed minimal COX-2 expression at high concentration.
- Troglitazone-induced COX-2 expression showed differential sensitivity to two distinct PI3K inhibitors.
- Furthermore, troglitazone-induced COX-2 expression was sensitive to the MEK inhibitor PD98059, to the p38 MAPK inhibitor SB202190 and to the PKC inhibitor Ro-32-0432.
- Neither troglitazone nor 15d-PGJ<sub>2</sub> convincingly induced I $\kappa$ B degradation, NF $\kappa$ B p65 phosphorylation, NF $\kappa$ B p65 activation or NF $\kappa$ B gene reporter activity.



- Minimal I $\kappa$ B degradation and NF $\kappa$ B p65 activation was seen at 24 hours post-treatment with Troglitazone, but these effects were not sensitive to the PPAR $\gamma$  antagonist, GW9662.
- Interestingly, the COX-2 product PGE<sub>2</sub> partially inhibited troglitazone induced COX-2 expression. However, the EP agonist sulprostone had no effect on troglitazone induced COX-2.
- Microarray analysis revealed differential regulation of gene expression by troglitazone and 15d-PGJ<sub>2</sub>.

#### Summary of section 4.2

- Troglitazone enhances TNF $\alpha$  induced COX-2 expression and PGE<sub>2</sub> production.
- Whereas 15d-PGJ<sub>2</sub> showed slight inhibitory effects on TNF $\alpha$  induced COX-2 expression but enhanced TNF $\alpha$  induced PGE<sub>2</sub> production.
- Troglitazone enhanced IL-1 $\beta$  induced COX-2 and 15d-PGJ<sub>2</sub> showed minimal inhibitory effects on IL-1 $\beta$  induced COX-2 expression.
- Another synthetic PPAR $\gamma$  ligand ciglitazone also enhanced TNF $\alpha$  and IL-1 $\beta$  induced COX-2 expression.
- Neither troglitazone nor 15d-PGJ<sub>2</sub> had any effect on TNF $\alpha$  induced I $\kappa$ B degradation, NF $\kappa$ B p65 phosphorylation or NF $\kappa$ B p65 activation.
- Troglitazone, but not 15d-PGJ<sub>2</sub>, inhibited TNF $\alpha$  induced NF $\kappa$ B gene reporter activity in a concentration dependent manner. This effect however was not reverse by the PPAR $\gamma$  antagonist GW9662.

- More selective PPAR agonists inhibited TNF $\alpha$  induced NF $\kappa$ B gene reporter activity at high concentrations.

## **4.4. Discussion II**

This chapter has investigated the disparate effects of two structurally distinct PPAR $\gamma$  ligands, namely troglitazone and 15d-PGJ<sub>2</sub>, on biochemical signalling pathways, COX-2 expression and gene expression in the lung epithelial cell line A549. I was unable to detect any convincing activation of NF $\kappa$ B by either of the PPAR $\gamma$  ligands. Moreover, use of more selective PPAR $\gamma$  agonist and antagonists revealed that the effects of troglitazone on COX-2 expression appeared to be independent of PPAR $\gamma$ .

### **Localisation of PPAR $\gamma$**

In the inactivated state, the PPARs are believed to be in complexes bound with corepressor proteins. In this state, in some but not all cell types, PPAR $\gamma$  may have a cytoplasmic rather than a nuclear location (Bishop-Bailey and Hla, 1999). Upon ligand activation, PPARs dissociate from corepressors and recruit coactivators and can translocate from the cytoplasm to the nucleus (Bishop-Bailey and Hla, 1999). Although PPAR $\gamma$  expression was restricted to the nucleus in A549 cell line, this is in line with other cell systems such as the human monocyte-derived macrophages (Chinetti et al., 1998).

### **PPAR $\gamma$ mediated signalling pathways**

With the aid of pharmacological inhibitors, I was able to determine the involvement of several signalling pathways in the induction of COX-2 by troglitazone, namely the PI3K and the ERK MAPK pathways. Troglitazone activated the PKB and ERK

pathways in a PI3K and MEK dependent manner, respectively. Troglitazone also induced COX-2 and mPGES expression and PGE<sub>2</sub> production. In comparison 15d-PGJ<sub>2</sub> activated the PI3K pathway, and had no effect on activation of the ERK pathway, induction of COX-2, mPGES or PGE<sub>2</sub> production. Both the PI3K and ERK MAPK pathways have been extensively shown to regulate COX-2 expression induced by a variety of cytokines (Matsuura et al., 1999; Chen et al., 2000; Weaver et al., 2001; Yang et al., 2002; Liu et al., 2003; Luo et al., 2003; Bradbury et al., 2004; Chen et al., 2004; Lin et al., 2004), however there is little previous evidence suggesting the involvement of these pathways in COX-2 expression induced by PPAR $\gamma$  ligands. Activation of signalling pathways such as the PI3K and ERK MAPK pathways are generally mediated via cell surface receptors. In this chapter I have shown that ligands of the nuclear receptor PPAR $\gamma$  can activate both pathways at time points that are associated with activation of membrane receptors. Previous studies in vascular smooth muscle cells have indicated that activation of PI3K and ERK pathway by PPAR $\gamma$  ligands to be mediated via novel membrane receptors (Takeda et al., 2001). Thus the disparate effects of troglitazone and 15d-PGJ<sub>2</sub> on signalling pathways explain the differential effects seen on functional outputs such as COX-2 and mPGES expression and PGE<sub>2</sub> production.

#### **Troglitazone induced COX-2 is mediated independently of PPAR $\gamma$**

Recently, bisphenyl A diglycidyl ether (BADGE) has been reported as a weak antagonist of PPAR $\gamma$  (Wright et al., 2000). Although BADGE inhibits adipocyte differentiation in cell culture, its use as an antagonist to elucidate the importance of PPAR $\gamma$  in biological processes may be limited by the low potency of binding. An

IC<sub>50</sub> of ~100 µM was reported, and incomplete inhibition of binding, apparently due to compound solubility, was observed (Wright et al., 2000). The limited solubility also complicates the interpretation of the receptor selectivity. By contrast, GW9662 is at least 10 000 times more potent than BADGE as measured by inhibition of [<sup>3</sup>H]Rosiglitazone binding to PPAR $\gamma$ , and an estimate of selectivity versus the other PPARs has been established (Leesnitzer et al., 2002). Mass spectrometric analysis of the PPAR $\gamma$  ligand binding domain treated with GW9662 established Cys<sup>285</sup> as the site of covalent modification. This cysteine is conserved among all three PPARs. In cell-based reporter assays, GW9662 was a potent and selective antagonist of full length PPAR $\gamma$ . The functional activity of GW9662 as an antagonist of PPAR $\gamma$  was confirmed in an assay of adipocyte differentiation (Leesnitzer et al., 2002). As the antagonistic activity of GW9662 towards PPAR $\gamma$  is retained in cell culture systems, this compound is a useful tool for elucidation of the role of PPAR $\gamma$  in biological processes. Complex behaviour has been reported for some PPAR $\gamma$  ligands, such as BADGE. This compound acts as an antagonist in some assays or cell lines, but as an agonist in others (Bishop-Bailey et al., 2000; Mukherjee et al., 2000). By contrast, GW9662 was a subtype-selective PPAR $\gamma$  antagonist under the conditions examined. The irreversible nature of GW9662 thus facilitates studies of PPAR $\gamma$  by enabling use of low doses and/or simplified dosing regimens. Despite the increased potency of GW9662 to PPAR $\gamma$  compared to BADGE, neither antagonist inhibited troglitazone induced COX-2 expression thus suggesting that this effect is mediated independently of PPAR $\gamma$ .

Other techniques may also be used for further work to verify that troglitazone-induced COX-2 expression is mediated independently of PPAR $\gamma$ . Such techniques

would involve the use of siRNA. The underlying molecular mechanism of gene silencing provides short interfering RNAs (siRNAs) which can target any gene with high specificity and efficiency (Schutze, 2004). A specific siRNA directed against PPAR $\gamma$  could be used to silence the gene and thus its expression. If troglitazone can still induce COX-2 expression following gene silencing this would further support the hypothesis that troglitazone induced COX-2 expression is mediated independently of PPAR $\gamma$ .

#### **Involvement of signalling pathways in Troglitazone induced COX-2 expression**

Troglitazone induced COX-2 expression was sensitive to the PI3K inhibitor LY294002 but insensitive to wortmannin. As suggested in Chapter 3, this could be due to the differential sensitivity of LY294002 and wortmannin to PI3K isoforms. However studies carried out with LY294002 in this chapter and work done by others suggests the role of PI3K in the regulation of COX-2 expression.

Recently, both PPAR $\alpha$  and  $\gamma$  ligands were shown to activate members of the MAPK family (Rokos and Ledwith, 1997; Mounho and Thrall, 1999; Gouni-Berthold et al., 2001; Lennon et al., 2002; Teruel et al., 2003). This event occurred at time points too rapid to account for new protein synthesis, suggesting that these agonists also exert PPAR-independent effects. Although these "nongenomic" effects of PPAR ligands are contradictory to the classical mechanism of nuclear receptor action, additional studies have illustrated that a variety of such compounds (*e.g.* progesterone, estrogen, and vitamin D) evoke similar rapid changes in kinase-mediated signal transduction pathways that contribute to their biological mechanism of action (Losel and Wehling, 2003). The involvement of the p38 MAPK pathway has also be recognised

downstream of troglitazone signalling (Gouni-Berthold et al., 2001; Bae and Song, 2003; Gardner et al., 2003). Moreover, *in vitro* and *in vivo* experiments have revealed that PPAR $\alpha$  and  $\gamma$  can be phosphorylated by some ( $\alpha$ ) or all three ( $\gamma$ ) MAPK leading to modulation of transcriptional activity (Hu et al., 1996; Camp et al., 1999; Juge-Aubry et al., 1999; Barger et al., 2001). Transcriptional changes induced by PPAR $\alpha$  and  $\gamma$  agonists can also be dissociated into distinct MAPK- and PPAR-dependent pathways (Baek et al., 2003), suggesting that MAPKs alone mediate some of the cellular effects of PPAR ligands. Thus, kinase activation by PPAR agonists appears to play an important role in the mechanism of action of these compounds; yet few studies have investigated how PPAR agonists stimulate MAPKs. In the study by Baek et al. (Baek et al., 2003), the authors reported that troglitazone but not other PPAR $\gamma$  ligands stimulated the expression of Egr-1. The authors also showed that troglitazone uniquely stimulated the ERK pathway that down-regulates the PPAR $\gamma$  receptor activity, indicating that the increased expression of Egr-1 was not mediated by the activity of PPAR $\gamma$  receptor (Chinetti et al., 1998; Baek et al., 2003). In terms of my findings, troglitazone induced COX-2 is sensitive to the MEK inhibitor PD98059, which is thought to be a specific inhibitor (Davies et al., 2000), and the p38 inhibitor SB202190 which lends further support to the involvement of the ERK and p38 MAPK pathway in troglitazone induced events. In addition, activation of the ERK pathway is known to phosphorylate and down-regulate PPAR $\gamma$  (Baek et al., 2003). Thus several mechanisms and pathways may appear to play an important role in the regulation of COX-2 by troglitazone.

There is little evidence of the involvement of the PKC pathway downstream of PPAR $\gamma$  ligand signalling. However, a study by Han & Sidell (2002), demonstrated

that PPAR $\gamma$  induced CD36 expression was dependent of PKC. In this chapter I have shown that PKC inhibitor Ro-32-0432 partially attenuated troglitazone induced COX-2 expression thus suggesting the involvement of this pathway downstream of cell stimulation with troglitazone. Furthermore, ligands of other nuclear receptors have also shown to activate the PKC pathway, however these effects have been suggest to be mediated via nongenomic actions of nonclassical receptors (Losel and Wehling, 2003).

### **Troglitazone induced COX-2 expression is not mediated via the classical NF $\kappa$ B pathway**

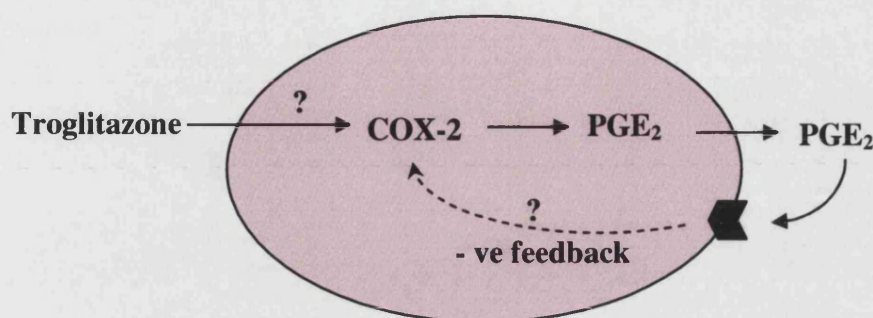
Involvement of the canonical NF $\kappa$ B pathway was the main NF $\kappa$ B pathway that was focussed on. Therefore the effects of troglitazone on other pathways leading to NF $\kappa$ B activation such as the NF $\kappa$ B1 p105 pathway can not be ruled out. Following cellular stimulation with agonists such as TNF $\alpha$ , IL-1 and LPS, p105 is phosphorylated and proteolysed by proteasomes (Mellits et al., 1993; Donald et al., 1995; Mackichan et al., 1996). Furthermore p105 is phosphorylated on serine 903 and 907 by glycogen synthase kinase (GSK)-3 $\beta$  with which it forms a complex and stabilizes p105 by preventing its degradation in unstimulated cells (Demarchi et al., 2003). TNF $\alpha$  induced proteolysis of p105 is blocked in GSK-3 $\beta$ -deficient fibroblasts or by induction of p105 S903A or S907A point mutations (Demarchi et al., 2003), suggesting this event may require p105 phosphorylation by GSK-3 $\beta$ . GSK-3 is a downstream target of activated PKB, and in this system I have shown activation of PKB following troglitazone stimulation. Thus activation of the NF $\kappa$ B1 p105 pathway may be involved in troglitazone induced COX-2 expression.



### **Regulation of Troglitazone induced COX-2 expression by PGE<sub>2</sub>**

The regulation of COX-2 by downstream products, such as PGE<sub>2</sub>, raises many issues. PGE<sub>2</sub> is shown to negatively regulate induced COX-2 protein at concentrations chosen because they reflect the amount of PGE<sub>2</sub> induced by cytokine stimulation. This supports the fact that these results are likely to be physiologically relevant with the induced PGE<sub>2</sub> production providing negative feedback for COX-2. This would effectively help limit the COX-2 dependent response (Figure 4.14). Evidence for a possible mechanism of action for this effect comes from recent work on the signalling pathways activated by PGE<sub>2</sub> (Sheng et al., 2001). A549 cell line has been shown to express EP3 and EP4 receptors (Yano et al., 2002). The binding of PGE<sub>2</sub> to its EP4 receptor, a G-protein receptor associated with an increase in cAMP following activation, has been shown to activate the PI3K signalling cascade and its downstream effector PKB. Evidence presented by Weaver et al., in HT29 cell line points to a negative regulatory role for PI3K on COX-2 expression (Weaver et al., 2001), thus providing a possible mechanistic pathway of action. However this has not been rigorously proven and PI3K does not seem to play a negatively regulatory role in this system. Furthermore, there is conflicting evidence from PGE<sub>2</sub> binding causing an increase in cAMP and a resulting positive effect on COX-2 expression (Fournier et al., 1997; Inoue et al., 2000). In addition, PGE<sub>2</sub> has been shown to stabilise COX-2 mRNA and stimulation of translation via a positive feedback loop mediated through the EP4 receptor and p38 MAPK in IL-1 $\beta$  stimulated human synovial fibroblasts (Faour et al., 2001). These hypotheses do not fit with the results presented here but may be playing a contributory role albeit with the net effect of inhibition of COX-2 by PGE<sub>2</sub>. Since the EP3 agonist sulprostone did not mimic the effects of PGE<sub>2</sub>, this data suggests that PGE<sub>2</sub> mediated inhibition of

troglitazone induced COX-2 is not via the EP3 receptor. Further investigation would be required to elucidate the involvement of the EP4 receptor.



**Figure 4.14.** Schematic representation of possible mechanism for PGE<sub>2</sub> negatively regulating troglitazone induced COX-2

Another possible explanation for the mechanism of action of troglitazone in inducing COX-2 may be through activation of other transcriptional pathways. Xie and Herschman (Xie and Herschman, 1995) were the first to demonstrate the importance of c-jun and CRE site for mediating the induction of COX-2. Moreover, AP-1 and the CRE were found to be important for the induction of COX-2 in human epithelial cells (Subbaramaiah et al., 1998; Subbaramaiah et al., 2000a; Subbaramaiah et al., 2000b). Thus the regulation of COX-2 expression by troglitazone may also involve the activation of the AP-1 pathway. In support of this hypothesis, microarray analysis showed a two-fold (or greater increase) significant increase in the gene expression of activating transcription factor 3 and 4- , which are components of the AP-1 family.

### **Troglitazone induces mPGES expression**

Troglitazone induced upregulation of mPGES and thus also PGE<sub>2</sub> production. The putative promoter of the human mPGES-1 gene (as described in Chapter 3) is GC-rich, lacks a TATA box, and contains binding sites for C/EBP  $\alpha$  and  $\beta$  and AP-1, two tandem GC- boxes, two progesterone receptor and three GRE elements (Naraba et al., 2002). As previously discussed, Egr-1 plays an important role in mPGES induction, and expression of this transcription factor can be upregulated by MAPK (Guha et al., 2001). In context of my findings, troglitazone was able to activate MAPK which may induce activation of Egr-1. Activated Egr-1 can bind to the proximal GC box in the mPGES-1 promoter, leading to transcriptional activation of the mPGES-1 gene (Murakami and Kudo, 2004). Recent evidence has indicated NF $\kappa$ B as another transcription factor important in mPGES expression (Catley et al., 2003), however since troglitazone was unable to activate NF $\kappa$ B, this transcription factor is not likely to play a role in troglitazone induced mPGES expression.

### **Disparate effect of troglitazone and 15d-PGJ<sub>2</sub> on regulation expression assessed by microarray analysis**

Despite the clear evidence that troglitazone induces COX-2 protein expression microarray analysis did not show a significant increase in mRNA expression of COX-2. COX-2 expression is detectable on the gene chips but following stimulation its expression does not vary much and is below the 2-fold cut off thus explaining why COX-2 expression is not on the genelists. Furthermore, the increase in protein expression could be due to altered turnover of COX-2, in that the protein accumulates because its degradation pattern has been affected.

There were only 2 genes that were shown to be regulated in the same way by troglitazone and 15d-PGJ<sub>2</sub> as assessed by microarray analysis. These genes were ribosomal protein S2 and heat shock 70kD protein (hsp 70) (highlighted in yellow in tables 4.2 and 4.3) and both these genes were downregulated following stimulation with either troglitazone or 15d-PGJ<sub>2</sub>. In addition to playing a role in the heat-shock response, in which hsp protect the cell from deleterious effects of an extreme change in the cell's environment i.e. high temperature, hsp 70 can act as molecular chaperones. However, hsp 70 has been shown to be upregulated by PPAR $\gamma$  ligands in rat islet cells (Weber et al., 2003). Furthermore, cyclopentenone PGs (including their precursor arachidonic acid) have been shown to activate heat shock stress response genes in different experimental models (Santoro et al., 1989; Koizumi et al., 1993; Jurivich et al., 1994; Weber et al., 2003). Ribosomal proteins are proteins that form complex catalytic machinery required for protein synthesis called ribosomes. Thus it would be expected that expression of ribosomal proteins would be upregulated following stimulation with PPAR $\gamma$  ligands since these ligands are known to regulate gene transcription, this however is not the case in A549 cells. The exact relevance of these findings remains unclear.

Several genes were upregulated by troglitazone and 15d-PGJ<sub>2</sub> and several downregulated by troglitazone, however it is difficult to assess the exact role of each of these genes and thus this next section will discuss the possible involvement of some of the more important genes (highlight in blue in table 4.3) in troglitazone induced events.

Upregulation of dual specificity phosphatases by troglitazone suggests a possible feedback mechanism by which troglitazone induces the expression of enzymes that can dephosphorylate proteins which may have been initially activated by troglitazone e.g. MAPK or transcription factors.

The upregulation of IL-8 was not surprising as previous studies have shown PPAR $\gamma$  ligands can activate IL-8 transcription and enhance IL-8 secretion in many cell systems including but not limited to lung cells (Jozkowicz et al., 2001; Zhang et al., 2001; Fu et al., 2002; Harris et al., 2002b). Contradictory to this, reports also document that PPAR $\gamma$  ligands can inhibit induced IL-8 expression in A549 cells (Trifilieff et al., 2003). It is difficult to interpret these results in the context of my findings since IL-8 expression was not evaluated following cytokine stimulation.

A shortage of oxygen in practically any type of cell, causes an increase in the intracellular concentration of the active form of a gene regulatory protein called hypoxia-inducible factor 1 (hif1). Hif can stimulate transcription of the vascular endothelial growth factor (VEGF). The microarray analysis detected upregulation of both hif and VEGF following stimulation with troglitazone, and expression of the latter gene may be dependent on the induction of hif.

Care must be taken evaluating microarray data as this method is limited in that the expression of a protein is only indicative of the transcriptional level and thus can not be used as a definitive measure of protein upregulation or down regulation. This may also explain some of the anomalies in the results obtained.

### **Debate over the selectivity of 15d-PGJ<sub>2</sub>**

15d-PGJ<sub>2</sub> has been employed in many studies to evaluate the role of PPAR $\gamma$  and in most cases it has shown to have effects similar to those of synthetic PPAR $\gamma$  ligands. One possible explanation for the disparate effects observed in this study between troglitazone and 15d-PGJ<sub>2</sub>, may be that 15d-PGJ<sub>2</sub> is rapidly degraded. Recently, the physiological and pharmacological relevance of 15d-PGJ<sub>2</sub> has been questioned. For example, 15d-PGJ<sub>2</sub> is thought to be highly reactive and covalently interacts with substances containing nucleophilic groups such as cysteinyl thiol groups of proteins including I $\kappa$ B kinase and H-Ras. Hence, the lack of selectivity for PPAR $\gamma$  may limit the usefulness of 15d-PGJ<sub>2</sub> as a tool for defining the role of this receptor. Moreover, the concentration required to activate PPAR $\gamma$  has been reported to be in the  $\mu$ M range, although it is doubtful whether 15d-PGJ<sub>2</sub> ever reaches these levels either in cultured cells or in *in vivo* models of inflammation (Bell-Parikh et al., 2003). Against this hypothesis is the fact that 15d-PGJ<sub>2</sub> showed effects on PI3K signalling pathway, TNF $\alpha$  induced COX-2 and PGE<sub>2</sub> expression as well as gene regulation.

### **Differential effects of troglitazone and 15d-PGJ<sub>2</sub> are mediated independently of PPAR $\gamma$ and NF $\kappa$ B**

The COX-2 promoter is known to contain a PPRE (Meade et al., 1999). Despite this, the selective PPAR $\gamma$  ligands investigated had no effect on COX-2 expression. Similarly, I could detect no NF $\kappa$ B activation by troglitazone, which is surprising given the role of NF $\kappa$ B in COX-2 expression is well recognised (Mitchell et al., 1994; Chen et al., 2000). These findings therefore indicate that troglitazone-induced regulation of COX-2 is independent of PPAR $\gamma$  in the systems used in this study.

This work lends further compelling evidence to the notion that troglitazone and 15d-PGJ<sub>2</sub> ligands can operate independently of PPAR $\gamma$ . For example previous studies using murine PPAR $\gamma$  null macrophages to demonstrate that previously reported inhibitory actions of PPAR $\gamma$  agonists on macrophage cytokine production occur via a PPAR $\gamma$ -independent mechanism (Chawla et al., 2001). Moreover, unique biochemical and functional actions of troglitazone which are not shared by 15d-PGJ<sub>2</sub> which may influence the therapeutic potential of this compound in inflammatory settings have been identified. This study also questions the potential of troglitazone related compounds as anti-inflammatory drugs given the robust upregulation of the pro-inflammatory mediator COX-2 by troglitazone.

#### **Effects of troglitazone on the TNF $\alpha$ induced NF $\kappa$ B pathway**

In addition to differential effects of the troglitazone and 15d-PGJ<sub>2</sub> alone, disparate effects were also seen with the two ligands on TNF $\alpha$ -induced events. The ability of troglitazone to inhibit TNF $\alpha$  induced NF $\kappa$ B gene reporter activity has previously been seen in adipocytes (Ruan et al., 2003). In my system this effect was shown to be independent of PPAR $\gamma$ , by the use of the PPAR $\gamma$  antagonists GW9662. Despite partial inhibition of NF $\kappa$ B reporter activity, pretreatment with troglitazone did not attenuate TNF $\alpha$  induced COX-2 or PGE<sub>2</sub> production, thus suggesting that the residual NF $\kappa$ B activity is sufficient for the induction of COX-2 expression and/or other transcription factors are responsible for the induction of COX-2 by TNF $\alpha$ .

**Regulation of cytokine induced COX-2 expression and activity by troglitazone and 15d-PGJ<sub>2</sub>**

PPAR ligands have been reported to regulate COX-2 expression, inducing COX-2 in human epithelial cells (Meade et al., 1999) but inhibiting its induction in LPS-stimulated differentiated U937 cells (Inoue et al., 2000). In addition, 15d-PGJ<sub>2</sub> has been shown to amplify COX-2 induction in response to stem cell factor, IL-1 $\beta$  and IL-10 in mouse bone marrow-derived mast cells, yet this effect was not seen to the same extent with synthetic PPAR $\gamma$  ligands (Diaz et al., 2002). The concept that 15d-PGJ<sub>2</sub> and troglitazone modulate the expression of proinflammatory genes differently has previously been shown (Petrova et al., 1999; Thieringer et al., 2000; Simonin et al., 2002). Several studies have identified new intracellular targets especially in the NF $\kappa$ B signalling pathway that are independent of PPAR $\gamma$  (Castrillo et al., 2000; Rossi et al., 2000; Straus et al., 2000). Importantly though, and in the context of the work presented here, little or no evidence has previously been reported on synthetic PPAR $\gamma$  ligands enhancing cytokine induced COX-2 expression in A549 cell line.

The role of TZDs in the treatment of Type II diabetes has been firmly established. The mechanism of action involves regulation of the expression of specific genes especially in fat cells but also other cell types such as endothelial cells, macrophages and monocytes, vascular smooth muscle cells and colonic epithelium by PPAR $\gamma$ . TZDs have been shown to interfere with expression and release of mediators of insulin resistance originating in adipose tissue (e.g., increased free fatty acids, decreased adiponectin) in a way that results in net improvement of insulin sensitivity (i.e., in muscle and liver). Interestingly there is an increase in COX-2 expression in pancreatic islet cells (Robertson, 1998) and COX-2 is thought to act as a negative



regulatory of insulin secretion. Although this study does not investigate the effects of troglitazone on COX-2 expression in islet cells it does highlight the potential side-effects (such as increased COX-2 and PGE<sub>2</sub> in lung epithelial cells) that may occur by patients taking TZDs for the treatment of Type II diabetes.

The cyclopentenone ring system of 15d-PGJ<sub>2</sub> can directly inhibit the DNA binding activity of NFκB through direct alkylation of a cysteine residue located in the DNA binding domain of the p65 subunit (Straus et al., 2000). This mechanism, in addition to inhibition of IKK, has been shown to act in combination to inhibit transactivation of the NFκB target gene COX-2 in Hela cells and RAW264.7 cells. However, in my system 15d-PGJ<sub>2</sub> was not able to inhibit IκB degradation, p65 phosphorylation or activation or NFκB reporter activity, thus suggesting that any inhibitory effect of 15d-PGJ<sub>2</sub> on cytokine induced COX-2 expression is not mediated via inhibition of the NFκB pathway.

The ability of 15d-PGJ<sub>2</sub> to enhance TNFα induced PGE<sub>2</sub> production may be due to inhibition of a NAD(+)-dependent 15-hydroxyprostaglandin dehydrogenase (PGDH) enzyme. PGDH is catabolic enzyme that controls the biological activities of prostaglandins by converting them into inactive keto-metabolites (Nandy et al., 2003). Studies have shown the distal element of PGDH, to be an integrator of transcriptional regulation by AP-1, Ets and CREB proteins (Greenland et al., 2000; Nandy et al., 2003). 15d-PGJ<sub>2</sub> has been shown to be able to directly inhibit AP-1 DNA binding by forming a covalent adduct with c-jun (Perez-Sala et al., 2003). In addition, anti-AP-1 activity of 15d-PGJ<sub>2</sub> has been shown to be a consequence of

inhibition of c-jun expression and competition for limiting amounts of the general coactivator CBP in a PPAR $\gamma$  dependent mechanism (Subbaramaiah et al., 2001).

The enhancement of cytokine induced COX-2 and PGE<sub>2</sub> by troglitazone and ciglitazone may simply be due to an additive effect. However what is of particular interest is that troglitazone, but not 15d-PGJ<sub>2</sub>, can inhibit TNF $\alpha$  induced NF $\kappa$ B reporter activity in a PPAR $\gamma$  independent mechanism. Furthermore, more specific PPAR ligands also produced the same effect albeit at concentrations far exceeding their EC<sub>50</sub> values. In support these finding are those by Trifilieff et al., who reported that selective PPAR $\alpha$ ,  $\gamma$  and  $\delta$  agonists inhibited the activation of NF $\kappa$ B-responsive gene construct at micromolar concentration (Trifilieff et al., 2003). This data suggests that synthetic PPAR ligands affect/inhibit a component of the NF $\kappa$ B pathway independently of PPAR. The possible mechanisms involved are discussed below.

Dimerization of the NF $\kappa$ B family members is necessary for their DNA-binding properties. Multiple homodimeric and heterodimeric combinations of these factors bind to consensus  $\kappa$ B sequences in the promoter element of NF $\kappa$ B regulated genes, resulting in unique patterns of transcriptional activation (Yamamoto and Gaynor, 2004). The p65-p50 heterodimer was defined as the classical NF $\kappa$ B-binding form, but this is only one of the multiple species that can bind to  $\kappa$ B sites. Although RelB homodimers do not bind to NF $\kappa$ B sites, heterodimers of this factor with either p50 or p52 can result in RelB-mediated transcriptional activation. Furthermore, studies have shown that RelA can be physically associated with both p100 and p105 as well as I $\kappa$ B $\alpha$  in the cytoplasm of cells (Sun et al., 1994; Dejardin et al., 1995). Sun et al.

(Sun et al., 1994), further showed that p100, like I $\kappa$ B $\alpha$ , potently inhibited the transcriptional activity of RelA.

Both p50 and p52 can also form transcriptionally active heterodimers with either p65 or c-Rel. p50 or p52 homodimers can also bind to NF $\kappa$ B sequences. In most tissues, these proteins have little intrinsic ability to activate transcription because they lack a classical transactivation domain. However, these homodimers can also repress the expression of their target genes (Li and Verma, 2002). For example, downregulation of cytokine-mediated NF $\kappa$ B activation can result from the replacement of the active p65-p50 heterodimer by the binding of p50 homodimers. The interaction of these homodimers with histone deacetylase complexes can then reduce cytokine-mediated gene expression (Li and Verma, 2002; Zhong et al., 2002).

Curiously, troglitazone inhibited TNF $\alpha$  induced NF $\kappa$ B reporter activity, although the significance of this finding remains unclear since troglitazone had no effect on other read-outs of TNF $\alpha$  stimulated NF $\kappa$ B activation. Thus this data also suggests that the residual effect of TNF $\alpha$  on NF $\kappa$ B is sufficient for the induction of COX-2 and/or that other transcriptional pathways are involved in TNF $\alpha$  induced COX-2 expression and in the additive effects of troglitazone on TNF $\alpha$  induced COX-2 expression. In support of the latter hypothesis is that troglitazone has been shown to increase AP-1 binding activity in rat synovial fibroblasts (Simonin et al., 2002). This also suggests that the decrease in TNF $\alpha$  induced NF $\kappa$ B reporter activity by troglitazone may not be the result of a physical interaction between proteins or between protein and DNA but rather the consequence of the titration of AP-1 and NF $\kappa$ B coactivators by the PPAR system. However, at this point, it is not possible to confirm this hypothesis.

#### **4.5. Conclusion**

The role of PPAR $\gamma$  ligands in the modulation of inflammation is nevertheless complex, because these ligands exhibit both anti-inflammatory and pro-inflammatory functions and thus remains the subject of intensive investigation. My findings therefore indicate that troglitazone-induced regulation of COX-2 is independent of PPAR $\gamma$  in the systems used in this study. This work lends further compelling evidence to the notion that troglitazone and 15d-PGJ<sub>2</sub> ligands can operate independently of PPAR $\gamma$ . For example previous studies using murine PPAR $\gamma$  null macrophages demonstrate that previously reported inhibitory actions of PPAR $\gamma$  agonists on macrophage cytokine production occur via a PPAR-independent mechanism (Chawla et al., 2001). Moreover, I have identified unique biochemical and functional actions of troglitazone that are not shared by 15d-PGJ<sub>2</sub>, which may influence the therapeutic potential of this compound in inflammatory settings (Patel et al., 2005) This study also questions the potential of troglitazone related compounds as anti-inflammatory drugs given the robust upregulation of the pro-inflammatory mediator COX-2 by troglitazone.

# **5.**

## **OVERALL DISCUSSION & CONCLUSIONS**

## **5. GENERAL DISCUSSION AND CONCLUSIONS**

### **5.1. General Discussion**

#### **5.1.1. Experimental Model**

The experimental model used in this work was *in vitro* culture of human lung epithelial cell line and the assessment of PPAR $\gamma$  ligands to modulate COX-2. In this context COX-2 appears to be behaving as an acute pro-inflammatory agent – induced by pro-inflammatory cytokines and synthetic PPAR $\gamma$  ligands and having its expression regulated by various signalling pathways. Interestingly different signalling pathways were involved in the regulation of COX-2 by cytokines and by the synthetic PPAR $\gamma$  ligand troglitazone. TNF $\alpha$ -mediated COX-2 expression showed involvement of ERK and p38 MAPK as well as the classical NF $\kappa$ B pathway. Similarly the involvement of the ERK and p38 MAPK pathways were important in troglitazone induced COX-2. In addition the PKC pathway played an important role in troglitazone induced COX-2 and these effects were independent of PPAR $\gamma$  and NF $\kappa$ B. Furthermore, this work shows disparate biochemical and functional effects between two PPAR $\gamma$  ligands (troglitazone and 15d-PGJ<sub>2</sub>) which were again mediated independently of PPAR $\gamma$ . Although allowing detailed manipulation of the regulatory pathways involved, the experimental model lacks the ability to pursue the more complex points. The system may also over-estimate the signalling pathways activated by TNF $\alpha$  and IL-1 $\beta$  and PPAR $\gamma$  ligands as transformed cell lines are believed to be more sensitive for activation of signalling cascades (Saklatvala et al., 1999).

One feature of the literature that is obvious is the heterogeneity in results obtained with PPAR $\gamma$  ligands between different models and cell types. There may be a common theme in the literature, such as TNF $\alpha$  or IL-1 $\beta$  inducing COX-2 which is shared in many systems. The conclusions outlined above are therefore from this work, rather than using other models which may not be relevant.

As seen in figure 3.18 there are many regulatory elements in an important gene such as COX-2 with the possibility that there may be interactions between them. There is also an element of redundancy and the possibility that stimulation may activate opposing signals. Such complexities make drawing detailed conclusions difficult. They also make the teasing out of the individual pathway effects challenging and often limited by the available molecular tools.

### **5.1.2. Comparison between troglitazone and 15d-PGJ<sub>2</sub>**

Troglitazone and 15d-PGJ<sub>2</sub> showed differential activation of signalling pathways and functional outputs. Troglitazone activated both the PI3K and MAPK pathway and induced COX-2 expression, in comparison 15d-PGJ<sub>2</sub> only activated the PI3K pathway and had no effect on basal or cytokine induced COX-2 expression. The COX-2 promoter is known to contain PPRE (Meade et al., 1999). Despite this, the selective PPAR $\gamma$  ligands investigated had no effect on COX-2 expression. Similarly, troglitazone did not induce NF $\kappa$ B activation which was surprising given that the role of NF $\kappa$ B in COX-2 expression is well recognized (Mitchell et al., 1994; Chen et al., 2000). Curiously, troglitazone inhibited TNF $\alpha$  induced NF $\kappa$ B reporter activity, although the significance of this finding remains unclear since troglitazone had no

effect on other readouts of TNF $\alpha$  stimulated NF $\kappa$ B activation. These findings therefore indicate that troglitazone-induced regulation of COX-2 is independent of NF $\kappa$ B and PPAR $\gamma$  this system.

### **5.1.3. Application to Lung Inflammatory Diseases**

The COX pathway is highly complex with multiple points of external and auto-regulation. Downstream prostaglandins, as well as upstream arachidonic acid liberation by phospholipases, will play a physiological role. The original hypothesis proposed was that PPAR $\gamma$  ligands may attenuate inflammatory responses by inhibition of pro-inflammatory genes such as COX-2. However this work questions the potential of troglitazone related compounds as anti-inflammatory drugs given the robust upregulation of the pro-inflammatory mediator COX-2 by troglitazone (Patel et al., 2005).



## **5.2. Overall Conclusions**

- The human lung epithelial cell line A549 expresses PPAR $\gamma$  and PI3K isoforms (Class IA and Class II).
- The PI3K inhibitors have differential selectivity for PI3K isoforms and thus this work has highlighted the potential pitfalls of using these inhibitors in elucidating the role of PI3K.
- Pro-inflammatory cytokines (TNF $\alpha$  and IL-1 $\beta$ ) activated PI3K, MAPK and NF $\kappa$ B signalling pathways which may be involved in their induction of COX-2 expression in the A549 cell line.
- Two structurally distinct PPAR $\gamma$  ligands have disparate effects on both basal and cytokine induced biochemical and functional events. These effects were shown to be PPAR $\gamma$  and NF $\kappa$ B independent.
- The PI3K, MAPK and PKC pathways play important roles in troglitazone induced COX-2 expression.
- The unique biochemical and function effects of troglitazone, which are not shared by 15d-PGJ<sub>2</sub>, may influence the therapeutic potential of this compound in inflammatory settings.

### **5.3. Future Work**

Future work may help to further identify the mechanisms by which PPAR $\gamma$  ligands act in an inflammatory response and thus further evaluate their potential as regulators of inflammation. These studies would be aided by further work described below:

- Supporting the work presented here with work in other models using alternative techniques. In particular, regarding the work based on PI3K inhibition, different techniques should be used such as molecular inhibition using dominant negative or constitutively active transfected constructs. Also dominant negative expression of PPAR (similar to that used by Gurnell et al., (2000) may be used to verify that the above described effects are PPAR $\gamma$  independent.
- This area of COX-2 research concentrates on the enzyme rather than its products, which are the focus of its functional activity. The relevance of troglitazone induced PGE<sub>2</sub> production warrants more research.
- Investigation into other transcriptional pathways (such as AP-1) pathway to evaluate the contribution of transcriptional pathways to troglitazone induced COX-2 expression.

# **6.**

# **APPENDIX**

## 6. APPENDIX

### 6.1. Buffers and solutions

#### 6.1.1 Solutions and buffers for SDS-PAGE and western blotting

<b>Lysis Buffer</b> 1% (v/v) Nonidet P-40, 150mM NaCl, 50mM Tris pH 7.5, 5mM EDTA, 10mM sodium fluoride* 1mM phenylmethylsulfonyl fluoride* 10µg ml <sup>-1</sup> leupeptin* 10µg ml <sup>-1</sup> aprotinin* 1µg ml <sup>-1</sup> soybean trypsin inhibitor* 1µg ml <sup>-1</sup> pepstatin A* 1mM sodium orthovanadate* 1mM sodium molybdate* MilliQ water	<b>4 x Resolving gel buffer</b> 1.5M Trizma base pH 8.8 0.4% (w/v) SDS MilliQ water  <b>4 x Stacking gel buffer</b> 0.5M Trizma base pH 6.8 0.4% (w/v) SDS MilliQ water
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NB \*denotes added on the day on use

<b>SDS-PAGE running buffer</b> 25mM Trizma base 192mM glycine 0.1% (w/v) SDS MilliQ water	<b>5X SDS-sample buffer</b> 5% SDS 50% glycerol 200mM Tris-HCl pH 6.8 MilliQ water Bromophenol blue 5% 2-mercaptoethanol
<b>Semi-dry transfer buffer</b> 39mM glycine 48mM Trizma base 0.0375% SDS 20% (v/v) methanol MilliQ water	<b>Tris-buffered saline (TBS)</b> 20mM Tris-HCl pH 7.5 150mM NaCl MilliQ water
<b>Tris-buffered saline- Tween (TBST)</b> TBS + 0.1% (v/v) Tween-20	<b>Stripping buffer</b> 62.5mM Tris-HCl pH 6.8 2% (w/v) SDS 100mM 2-mercaptoethanol MilliQ water

### 6.1.2. Recipes for various percentage SDS page gels

Final percentage gel	7.5%	10%	12%	Stacking gel	
<b>4x Resolving buffer</b>	5.0ml	5.0ml	5.0ml	<b>4x Stacking buffer</b>	3.0ml
<b>MilliQ</b>	9.84ml	8.17ml	6.84ml		6.85ml
<b>Acrylamide</b>	5.0ml	6.67ml	8.0ml		2.0ml
<b>APS (10%)</b>	150µl	150µl	150µl		150µl
<b>TEMED</b>	15µl	15µl	15µl		15µl

**6.1.3. Buffers for Nuclear Extract Preps**

Buffer 1	Buffer 2
10mM HEPES	10mM HEPES
10mM KCl	400mM NaCl
100µM EDTA	100µM EDTA
100µM EGTA	100µM EGTA
1mM Sodium molybdate*	1mM Sodium molybdate*
1mM Sodium vanadate*	1mM Sodium vanadate*
10mM Sodium fluoride*	10mM Sodium fluoride*
MilliQ water	MilliQ water

NB \*denotes added on the day on use

**6.1.4. Buffers for *in vitro* lipid kinase assay**

<b>PBS + 1% NP-40</b> 137mM NaCl 2.7mM KCl 8mM Na <sub>2</sub> HPO <sub>4</sub> 1.5mM KH <sub>2</sub> PO <sub>4</sub> 0.01% NP-40 100µM Sodium orthovanadate * PBS	<b>Tris/LiCl Buffer</b> 100mM Tris pH7.4 5mM LiCl 100µM Sodium Orthovanadate * MilliQ
<b>TNE Buffer</b> 10mM Tris 150mM NaCl 5mM EDTA 100µM Sodium orthovanadate * MilliQ	<b>ATP Kinase buffer</b> 0.88mM ATP (ATP made up in 10mM Tris HCl pH7.4) 20mM MgCl <sub>2</sub> 5µCi (γ <sup>32</sup> P)-ATP 10mM Tris HCl pH7.4
<b>Preparation of PI</b> -Appropriate amount of PI was dried under nitrogen. 10mM Tris pH 7.4, 1mM EDTA was added to give a concentration of 2mg/ml. PI was resuspended by sonicating in an ice bath for 10mins	

NB \*denotes added on the day on use

**6.2. Antibodies and conditions used for immunoblotting**

Primary antibody	Mwt (kDa)	Blocking buffer (made up in TBST)	Dilution of primary Ab (in TBST + 0.01% azide)	Secondary antibody	Dilution of secondary antibody
Cyclooxygenase 2	74	5% Marvel	1:2000 in 1% Marvel	Anti-rabbit HPR	1:10,000 in 2.5% Marvel
Cyclooxygenase 1	68	5% Marvel	1:1000 in 1% Marvel	Anti-goat HPR	1:10,000 in 1% Marvel
mPGES	16	5% Marvel	1:1000	Anti-rabbit HRP	1:7000 in 1% Marvel
I $\kappa$ B	37	5% Marvel	1:1000	Anti-rabbit HRP	1:10,000 in 1% Marvel
NF $\kappa$ B phospho-p65 <sup>Ser276</sup>	65	5% Marvel	1:1000	Anti-rabbit HRP	1:7,000 in 1% Marvel
Pan PKB	60	5% Marvel	1:1000	Anti-goat HRP	1:10,000 in 1% Marvel
Phospho <sup>Ser473</sup> PKB	60	1% Marvel	1:1000	Anti-rabbit HPR	1:7,000 in 1% Marvel
Pan ERK	42/44	5% Marvel	1:1000	Anti-rabbit HPR	1:10,000 in 1% Marvel
Phospho <sup>Thr202/Tyr204</sup> ERK 1/2	42/44	5% Marvel	1:1000	Anti-rabbit HPR	1:10,000 in 1% Marvel
PPAR $\gamma$	54	5% Marvel	1:500	Anti-rabbit HPR	1:5,000 in 5% Marvel
PI3K-C2 $\alpha$	180	5% Marvel	1:1000	Anti-rabbit HPR	1:10,000 in 5% Marvel
PI3K-C2 $\beta$	180	5% Marvel	1:1000	Anti-rabbit HPR	1:10,000 in 5% Marvel
p85	85	5% BSA	1:1000	Anti-mouse HPR	1:20,000 in 2.5% BSA
p110 $\gamma$	110	5% BSA	1:1000	Anti-goat HPR	1:20,000 in 2.5% BSA

**7.**

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